

Bacillus

Edited by
Colin R. Harwood

*The University of Newcastle upon Tyne
Newcastle upon Tyne, United Kingdom*



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Bacillus

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Preface

The genus *Bacillus* has a long history of importance, both from an economic point of view and as a source of experimental microorganisms. This volume critically reviews aspects of identification, molecular biology, and growth that are of importance for the current and anticipated future exploitation of members of this group. In addition, the volume includes a chapter on taxonomy, as the importance of good taxonomy is often not fully appreciated; on sporulation, since so many important products are produced concomitantly with this process and we are beginning to understand the mechanisms by which the process is controlled; and, finally, on the cell envelope, as we are only just beginning to appreciate the significance of differences between the cell walls of gram-positive and gram-negative bacteria for productivity and processing.

The commercial importance of *Bacillus* lies mainly in the area of enzyme production for the food, drink, and detergent markets. Increasingly, however, the ability of *Bacillus* to secrete proteins, coupled with its regulatory acceptability, has resulted in strenuous efforts to develop species of *Bacillus* as hosts for the production of value-added heterologous proteins. Difficulties have often been encountered, indicating a need to divert more resources to improving our understanding of the molecular biology of members of this group. Experience with *Escherichia coli*, a far from ideal organism from a commercial point of view, suggests that an increased investment in *Bacillus* is likely ultimately to be productive. An important stimulus would be the successful outcome of current plans for sequencing the entire genome of *B. subtilis*.

This volume has been conceived primarily with three groups of scientists in mind: *Bacillus* specialists looking to gain an insight into other aspects of the biotechnology of this genus, academic and industrial researchers requiring a critical account of the relative advantages of using *Bacillus* for new applications, and senior students requiring a broadly based review of the biotechnology of *Bacillus*.

Finally, this book is dedicated to Donald Dean and his collaborators at the *Bacillus* Genetic Stock Center, Ohio State University, in appreciation of their outstanding service to the *Bacillus* scientific community.

Colin R. Harwood

Newcastle upon Tyne

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Introduction to the Biotechnology of *Bacillus*

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Bacteria of the genus *Bacillus* are aerobic, endospore-forming, gram-positive rods. The genus was created in 1872 by Ferdinand Cohn, who changed the name of Ehrenberg's (1835) "*Vibrio subtilis*" to *Bacillus subtilis*. The Cohn strain, *B. subtilis* Cohn 1872, 174, remains the type species of the genus.

The genus is one of the most diverse and commercially useful groups of micro-organisms. Representatives of this genus are widely distributed in soil, air, and water where they are involved in a range of chemical transformations that rival those of the Pseudomonads and Actinomycetes (see Chapter 3). Although primarily saprophytes, at least one species (*B. schlegelii*) shows facultative chemolithotrophic activity in an $O_2/CO_2/H_2$ or O_2/CO_2 atmosphere (Schenk and Aragno, 1979) and two species (*B. macerans* and *B. polymyxa*) are able to fix nitrogen (Hino and Wilson, 1958; Witz *et al.*, 1967). The ability of certain strains to tolerate high or low temperature and high and low pH has made them a particularly important source of commercial enzymes (Norris *et al.*, 1981).

The metabolic diversity of *Bacillus* spp., together with low reported incidence of pathogenicity, has led to representatives of this group being used in a wide range of industrial processes. One of the oldest recorded uses is the fermentation of soybeans into natto, a tempe-like fermentation that uses a strain of *Bacillus* now recognized as a variant of *B. subtilis*. Some 6×10^6 kg of natto are consumed annually in Japan (Djen and Hesseltine, 1979), surely the finest testimony to the suitability of this organism for the production of products for the food and pharmaceutical industries. Nowadays strains of *Bacillus* are used for the production of four main types of product: (1) enzymes, (2) antibiotics, (3) fine biochemicals including flavor enhancers and food supplements, and (4) insecticides (see Chapters 10 and 11).

It is the enzyme market that currently accounts for the main commercial importance of members of the genus. In particular they are the major source of hydrolyzing enzymes for the food and detergent markets. Subtilisin, an alkaline protease isolated from *B. subtilis* and closely related species, represents some 25% of

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the world enzyme market and is calculated to be worth about \$26 million per annum in the United States alone (Towalski and Rothman, 1986). The continual requirement of the detergent industry for proteases with greater heat and alkaline tolerance has resulted in two quite separate approaches to the production of improved enzymes. The more traditional approach has been to isolate organisms from increasingly alkaliphilic and thermophilic environments. Although this approach is initially very successful, the law of diminishing returns means that the rate of discovery of "improved" enzymes decreases with time, particularly when conventional screening programs are employed. However, we are now beginning to see the wider application of modern selective isolation techniques in which a large amount of phenetic data, accumulated in computer databases, are employed both to devise the selection regimes and to analyze the resulting isolates for genuine "novelty." To exploit these newer methods to the full, progress will need to be made on some of the problems currently besetting *Bacillus* taxonomy (see Chapter 2).

The alternative approach to improving enzyme performance, and one that has exciting possibilities for many other systems, is that of enzyme engineering. This approach involves engineering precise changes in the nucleotide sequence encoding the enzyme in question to alter particular amino acid residues in its primary sequence. Fersht and colleagues (Thomas *et al.*, 1985), for example, studied the effects of changes in surface charge on the pH dependence of subtilisin BPN' by making individual amino acid substitutions that influence the protonation of His64 at the active site of this enzyme.

The production of microbial pesticides like the delta-endotoxins produced during sporulation by *B. thuringiensis*, *B. popilliae*, and *B. lentimorbus* represents a potentially elegant solution to increasing resistance to chemical pesticides and their toxic effects in man and the environment. The production of microbial pesticides is currently low, worth at most some \$10 million per annum compared with some \$13 billion for chemical alternatives (Hacking, 1986). However, it is to be hoped that their particular advantages of lack of general toxicity to man and the environment, their lack of persistence, and their specificity for particular insect pests will outweigh their significant cost disadvantage in an increasingly environmentally conscious world.

More recently, with the development of a variety of host/vector systems (see Chapters 6 and 7), recombinant DNA technology has expanded considerably the potential commercial importance of *B. subtilis* and other representatives of the genus. This point was first brought home to me by Frank Young (1980), appropriately in the Eighth Griffith Memorial Lecture of the Society for General Microbiology. Until this time I, and probably countless others, had regarded *B. subtilis* primarily as an amenable "model" microorganism in which to study the physiology and molecular biology of such processes as sporulation and hadn't foreseen its potential to rival *Escherichia coli* as a commercial producer of the products of genetic engineering.

Our understanding of the genetics and physiology of *B. subtilis* is second only to that of *E. coli* and surely will be the second living organism to have its entire nucleotide sequence determined. The adoption of this organism for so many investigations relates directly to the pioneering work of Spizizen (1958), whose demonstration of transformation in *B. subtilis* was the first for any nonpathogenic microorganism. Exploitation of transformation together with other subsequently

developed genetic systems, most notably PBS1-mediated transduction which packages up to 8% of the bacterial genome, has lead to the development of a well-defined genetic map of over 400 loci (Piggot and Hoch, 1985; see Chapter 4).

The metabolism of *Bacillus* adapts to a lack of continuity of nutrients in a variety of ways, most significantly by secreting various hydrolytic enzymes and by producing heat-, radiation-, and desiccation-resistant endospores that may survive in a dormant state for many years if not centuries (Sneath, 1962; Seaward *et al.*, 1976). It is interesting to note that endospore formation was first observed as long ago as 1876 by Cohn (*B. subtilis*) and by Koch (*B. anthracis*). These spores, and the multistage process by which they develop, have been the subject of intense study both because of their importance in the food-processing and sterile products industries, and because they represent a model for cellular differentiation. Although it is tempting to regard bacterial sporulation as a *simple* form of differentiation, the process has been shown to be extremely complex, in terms of both its biochemistry and the mechanisms that have evolved to control it (see Chapter 8). Unraveling the complexities of this process has required great ingenuity on the part of the experimenters. The "cascades of sigma factors" that control sequential gene expression during sporulation in *B. subtilis* have proved to be a very useful model for studying morphological development in *Streptomyces* spp. and, since production of secondary metabolites occurs during this differentiation process, this holds out the prospect of controlling and improving the productivity of this important group of industrial microorganisms.

Sporulation and the other metabolic changes that occur at the end of exponential growth are very important in the productivity of *Bacillus* spp. It is at this time that the synthesis of many of its commercially significant proteins are induced. This applies not only to the production of enzymes like α -amylase and proteases from *B. amyloliquefaciens*, *B. licheniformis*, and *B. subtilis*, but also to the insecticidal toxins synthesized by *B. thuringiensis*, *B. larvae*, *B. lentimorbus*, and *B. popilliae* (see Chapters 10 and 11). The mechanisms that control both the synthesis and, in the case of the exoenzymes, the secretion of these products are being developed for the production of heterologous proteins from *Bacillus* (see Chapter 5). One of the properties of *Bacillus* spp. that makes them so attractive for commercial processes is the ability to secrete high levels of protein into the growth medium, up to 12 g/liter in the case of the S protein of *B. brevis* (Ohmiza *et al.*, 1983; see Chapters 5 and 9). This may ultimately confer an important competitive advantage for the production of bulk pharmaceutical products like human serum albumin or veterinary products like somatotrophin.

Apart from the well-established pathogenicity of *B. anthracis*, a pathogen of humans and some animals, *B. cereus*, which causes gastroenteritis, and the group of insect pathogens related to *B. thuringiensis*, most other species of *Bacillus* are regarded as nonpathogenic or cause only opportunistic infections, often in compromised patients. The lack of pathogenicity among strains of *B. subtilis* or any of its close relatives has resulted in the Food and Drug Administration granting the organism GRAS (generally regarded as safe) status.

The prospects for the continued commercialization of the *Bacillus* are good. The excellent genetic and physiological systems have permitted, for example, the isolation of mutants that produce glutamic acid as cost effectively as *Corynebacterium glutamicum*. Genetic engineering has increased significantly the productivity of

Bacillus for certain traditional products such as α -amylase, and is likely to do the same for other enzymes, required in bulk quantities, that are products of members of this genus. However, with a very few exceptions, *Bacillus* has not yet proved a suitable host for high-level production of heterologous proteins, e.g., factor VIII, interferon, etc. Problems have been experienced with proteolytic digestion, although these are likely to be solved by the isolation of improved protease-negative strains. Nevertheless, unforeseen problems have been experienced in exporting heterologous proteins, the solution to which is likely to require a much greater understanding of the secretion process and the involvement of components of the cell envelope.

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Taxonomy of *Bacillus*

2

DIETER CLAUS and DAGMAR FRITZE

1. GENERAL ASPECTS

A century ago, when a young scientist detected that a bacterium which Pasteur had thought to be a coccus was in fact a short rod, Pasteur's comment was, "I wish I could convey to you how little that information excites me" (Duclaux, 1896). Obviously, Pasteur was interested in microorganisms mainly for what they do rather than what they are. Whereas this remains a legitimate interest today, early bacteriologists felt that, with the increasing diversity of organisms isolated, there was a need to arrange bacteria distinctly. The easiest way to do this was to put them into groups with common properties, to study the relationships of such groups and their role in nature, and to develop methods that would enable scientists to identify new isolates with already known groups.

Such studies belong to the field of systematics, a term related to and often used interchangeably with taxonomy. Taxonomy, like systematics, is concerned with the classification or systematic arrangement of organisms into groups or categories called taxa (singular: taxon). It can be divided into three parts:

1. Classification, which is the arrangement of organisms into groups (taxa) on the basis of similarities or relationships.
2. Nomenclature, which is the assignment of names to taxonomic groups according to international rules.
3. Identification, which is the process of determining whether a new isolate belongs to one of the taxa defined and named by 1 and 2.

With bacteria, classification is carried out in a number of stages. The practical working unit is a pure culture* of an isolated bacterium known as a "strain."

*A culture is a population of bacterial cells in a given place at a given time. A pure culture is ideally the progeny of one cell. Pure cultures are generally obtained by plating and replating a suspension of a single, well-isolated colony developing on a solidified nutrient medium assuming (!) that a discrete colony has developed from the multiplication of a single cell. The term clone is often used synonymously with pure culture. It should be restricted to those pure cultures which, by use of such special techniques as the use of a micromanipulator, are really known to have developed from a single cell.

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Related strains are grouped to species (e.g., *Bacillus subtilis*), the latter to genera (singular: genus, e.g., *Bacillus*), and these to families (e.g., *Bacillaceae*).

Classification may have the purely utilitarian aim of subsequently simplifying the process of identifying new strains. In contrast to this "artificial" classification the "natural" or phylogenetic classification attempts to develop a classification system based on species thought to have evolved in separate lines from a common ancestor. Great progress in phylogenetic classification has been made in recent years by studying nucleic acid sequences (Stackebrandt *et al.*, 1987).

Not every scientist is aware of the fact that systematics and classification, in general, are disciplines underlying any scientific work. It would hardly be possible to make any generalization on bacteria and their role in nature if we could only refer to each isolate by a different and arbitrary name. However, from the early period of bacteriology, taxonomy has seen many changes. Nontaxonomists have always been worried by new classification systems and nomenclatural changes, forgetting that scientific concepts in all other fields of bacteriology have also undergone and will continue to undergo changes. Some of them tend to agree with Cowan (1978), who feels that a species may be generally considered as a group of organisms defined more or less subjectively by the criteria chosen by a taxonomist to show to best advantage, and as far as possible to put into practice, his individual concept of what a species is.

The practical problems in bacterial classification are (1) adequate characterization of a strain, (2) the process of comparison of strains, and (3) the determination of the boundaries of the various taxa.

In nomenclature we can rely on the Bacteriological Code, which is the short title of the International Code of Nomenclature of Bacteria (Lapage *et al.*, 1975). It includes established principles, rules, and recommendations and, if followed by all bacteriologists, will reduce nomenclatural difficulties. However, there is still disagreement regarding the above-mentioned classification problems. Undoubtedly, a soundly based classification system depends on basic agreements and on a more universally accepted species concept which should not undergo permanent changes.

It is one of the tasks of the International Committee on Systematic Bacteriology to seek more agreement for classification problems and, indeed, the situation has improved considerably in recent years. This is due mainly to the extension of strain or species descriptions, which now generally also include data like DNA base composition, DNA/DNA homology, or data from chemotaxonomic studies, and by using more standardized methods for the phenotypic description of strains. Aspects of the classification and nomenclature of the genus *Bacillus* will be presented below. The identification of newly isolated *Bacillus* strains requires adequate characterization, description, and comparison with descriptions of validly published *Bacillus* species. These aspects are discussed in Chapter 3.

2. TAXONOMY OF THE GENUS *BACILLUS*

2.1. Historical Aspects

The generic name *Bacillus* was first established in 1872 by Ferdinand Cohn to include rod-shaped bacteria that grow in filaments. In 1876, Cohn and Koch inde-

pendently detected that two of the three recognized species of this genus, *B. subtilis* and *B. anthracis*, form a special structure, the bacterial endospore or spore, which is highly resistant to heat and cannot be killed easily by mere boiling. Also the development from cell to resistant spore and from spore to vegetative cell was demonstrated.

The use of the unusual ability of spore formation as a character in keys for classification of bacteria was first proposed in 1884 by de Bary. In his key the genus *Bacillus* was for the first time restricted to endospore-forming bacteria. Other microbiologists, however, continued to use the generic name *Bacillus* for bacterial groups defined as (1) any rod-shaped bacterium; (2) rod-shaped bacteria occurring in filaments; (3) rods motile by means of peritrichously inserted flagella; or (4) any motile rod (Buchanan, 1970).

It is not surprising that the assignment of so many rod-shaped bacteria to the genus *Bacillus* resulted in a profusion of species. *Index Bergeyana* and its supplement (Buchanan *et al.*, 1966; Gibbons *et al.*, 1981), which covers nearly all bacterial names ever used, lists more than 8000 *Bacillus* species; most of them have now been assigned to other well-established genera.

It was only from about 1920 that the diagnostic importance of bacterial endospore formation was generally accepted. From then on the genus *Bacillus* was defined to include all endospore-forming, rod-shaped, straight or nearly straight bacteria capable of growing aerobically.

Since the general recognition of the genus *Bacillus* as defined above, many new *Bacillus* species have been described. However, species descriptions were often poor, the extent of strain variation was not always fully appreciated, and "comparative work necessary to put an organism into its rightful place in an existing genus or species" (Cowan and Steel, 1974) was in most cases neglected.

The first comprehensive study on the taxonomy of the genus *Bacillus* was done by Smith *et al.* (1946), followed by an updated work 6 years later (Smith *et al.*, 1952). This pioneering work in bacterial taxonomy was based on the comparative study of 1134 strains bearing 179 different species names or which were designated only as *Bacillus* sp. By studying about 35 morphological, physiological, and biochemical properties, most of the strains could be assigned to just 19 species.

The taxonomic studies of Smith *et al.* (1946, 1952), continued by Gordon *et al.* (1973), lead to a classification similar to that in the latest edition of *Bergey's Manual of Determinative Bacteriology* (Gibson and Gordon, 1974). In addition to 22 more widely accepted *Bacillus* species, the *Manual* also includes 26 species of arguable status considered to need further studies to confirm their standing in nomenclature.

2.2. Approved Lists of Bacterial Names

A tremendous number of bacterial species had been described before any rules of nomenclature had been developed. As a result the same bacterium could be classified with differently named taxa. To avoid this, all priorities of bacterial names back to May 1, 1753 were determined under the International Code of Nomenclature. This meant that it was no longer permissible to use a name already given to an organism for another organism of the same rank or within the same genus. In 1973, the Judicial Commission of the International Committee on Systematic Bacteriology appointed an ad hoc committee to organize a review of the currently valid names of the bacteria. Their object was to retain only names for bacteria which are

Table I. Validly Described *Bacillus* Species and Collection Numbers of the Type Strains

Name	Author and year	ATCC	CCM	DSM	NCIB	NCTC	NRRL
<i>Bacillus acidocaldarius</i>	Darland and Brock, 1971 ^a	27009		446	11725		
<i>Bacillus alcalophilus</i>	Vedder, 1934 ^a	27647		485	10436	4553	
<i>Bacillus alginolyticus</i>	Nakamura, 1987 ^b			5050			NRS-1347
<i>Bacillus alvei</i>	Cheshire and Cheyne, 1885 ^a	6344	2051	29	9371	6352	
<i>Bacillus amyloliquefaciens</i>	Priest <i>et al.</i> , 1987 ^b	23350		7			
<i>Bacillus amylolyticus</i>	Nakamura, 1984a ^b	9995		3034			NRS-290
<i>Bacillus anthracis</i>	Cohn, 1872 ^a	14578			9377	10340	
<i>Bacillus azotofixans</i>	Seldin <i>et al.</i> , 1984 ^b	35681			12093		
<i>Bacillus azotoformans</i>	Pichinoty <i>et al.</i> , 1983 ^b	29788	2849	1046	11859		
<i>Bacillus badius</i>	Batchelor, 1919 ^a	14574	2113	23	9364	10333	
<i>Bacillus benzoovorans</i>	Pichinoty <i>et al.</i> , 1987 ^b (Effect. Publ.: Pichinoty <i>et al.</i> , 1984)						
<i>Bacillus brevis</i>	Migula, 1900 ^a	8246	2050	30	9372	2611	
<i>Bacillus cereus</i>	Frankland and Frankland, 1887 ^a	14579	2010	31	9373	2599	
<i>Bacillus chondroitinus</i>	Nakamura, 1987 ^b			5051			NRS-1351
<i>Bacillus circulans</i>	Jordan, 1890 ^a	4513	2048	11	9374	2610	NRS-726
<i>Bacillus coagulans</i>	Hammer, 1915 ^a	7050	2013	1	9365	10334	NRS-609
<i>Bacillus fastidiosus</i>	Den Dooren De Jong, 1929 ^a	29604		91	11326		
<i>Bacillus firmus</i>	Bredemann and Werner, 1933 ^a	14575	2213	12	9366	10335	
<i>Bacillus globisporus</i>	Larkin and Stokes, 1967 ^a	23301	2119	4	11434		
<i>Bacillus gordonae</i>	Pichinoty <i>et al.</i> , 1986 ^b	29948					
<i>Bacillus insolitus</i>	Larkin and Stokes, 1967 ^a	23299	2175	5	11433		
<i>Bacillus larvae</i>	White, 1906 ^a	9545					
<i>Bacillus laterosporus</i>	Laubach, 1916 ^a	64	2116	25	9367	6357	NRS-666
<i>Bacillus lautus</i>	Nakamura, 1984a ^b			3035			B-2522
<i>Bacillus lentimorbus</i>	Dutky, 1940 ^a	14707		2049			
<i>Bacillus lentus</i>	Gibson, 1935 ^a	10840	2214	9	8773	4824	
<i>Bacillus licheniformis</i>	Chester, 1901 ^a	14580	2145	13	9375	10341	

<i>Bacillus macerans</i>	Schardinger, 1905 ^a	8244	2012	24	9368	6355
<i>Bacillus macquariensis</i>	Marshall and Ohye, 1966 ^a	23464		2	9934	10419
<i>Bacillus marinus</i>	Rüger, 1983 ^b	29841		1297		
<i>Bacillus megaterium</i>	De Bary, 1884 ^a	14581	2007	32	9376	10342
<i>Bacillus mycoides</i>	Flügge, 1886 ^a	6462		2048		
<i>Bacillus pabuli</i>	Nakamura, 1984a ^b			3036		NRS-924
<i>Bacillus pallidus</i>	Scholz <i>et al.</i> , 1988 ^b (Effect. Publ.: Scholz <i>et al.</i> , 1987)			3670		
<i>Bacillus pantothenicus</i>	Proom and Knight, 1950 ^a	14576	2049	26	8775	8162
<i>Bacillus pasteurii</i>	Chester, 1898 ^a	11859	2056	33	8841	4822
<i>Bacillus polymyxa</i>	Macé, 1889 ^a	842	1459	36	8158	10343
<i>Bacillus popilliae</i>	Dutky, 1940 ^a	14706		2047		
<i>Bacillus psychrophilus</i>	Nakamura, 1984b ^b	23304	2117	3		B-2309
<i>Bacillus pulvificiens</i>	Nakamura, 1984c ^b			3615		NRS-1530
<i>Bacillus pumilus</i>	Meyer and Gotheil, 1901 ^a	7061	2144	27	9369	B-3685
<i>Bacillus schlegelii</i>	Schenk and Aragno, 1981 ^b (Effect. Publ.: Schenk and Aragno, 1979)			2000		
<i>Bacillus smithii</i>	Nakamura <i>et al.</i> , 1988 ^b			4216		NRS-173
<i>Bacillus sphaericus</i>	Meyer and Neide, 1904 ^a	14577	2120	28	9370	10338
<i>Bacillus stearothermophilus</i>	Donk, 1920 ^a	12980	2062	22	8923	10339
<i>Bacillus subtilis</i>	Cohn, 1872 ^a	6051	2216	10	3610	3610
<i>Bacillus thermoglucosidasius</i>	Suzuki, 1984 ^b (Effect. Publ.: Suzuki, 1984)			2542	11955	
<i>Bacillus thermoruber</i>	Manachini <i>et al.</i> , 1985 ^a					
<i>Bacillus thuringiensis</i>	Berliner, 1915 ^a					
<i>Bacillus tusciae</i>	Bonjour and Aragno, 1985 ^b (Effect. Publ.: Bonjour and Aragno, 1984)	10792	19	2046	9134	
<i>Bacillus validus</i>	Nakamura, 1984a ^b			3037		NRS-1000

^a AL: Approved Lists of Bacterial Names (Skerman *et al.*, 1980), see Section 2.2.

^b VP: Validly Published Names, Validation of the Publication of New Names and New Combinations Previously Effectively Published Outside the International Journal of Systematic Bacteriology, see Section 2.2.

adequately described and for which there is a type or neotype strain described, and to compile these names under the title "Approved Lists of Bacterial Names." These lists have been published in the *International Journal of Systematic Bacteriology (IJSB)* and became effective on January 1, 1980 (Skerman *et al.*, 1980).

It is important that only names that appeared on the Approved Lists need now to be taken into consideration and January 1, 1980 replaces the year 1753 as the date for recognition of priority of new names. Those names published prior to 1980 but not included in the Approved Lists are not further recognized. They have no further standing in nomenclature. Such names, however, are available for reuse for the naming of new taxa. From the date of publication of the Approved Lists, a name of a new taxon is not validly published unless it has been published in the *IJSB* either in the form of an original publication or by inclusion in one of the Validation Lists of the *IJSB*.

2.3. Validly Published *Bacillus* Species

Based on the classification system of Gordon *et al.* (1973), the Approved Lists include 32 *Bacillus* species. Volume 2 of the new *Bergey's Manual of Systematic Bacteriology* will include 34 *Bacillus* species (Table I). In addition, 26 species are listed as *species incertae sedis*. Their names are now invalid but may be considered for revival after more detailed studies or after isolation and examination of additional strains (Claus and Berkeley, 1986). Some of the strains within this group of invalidly named species are extensively used in scientific studies. If cited, their names should be given in quotation marks only (e.g., "*Bacillus caldolyticus*"). After completion of the manuscript for *Bergey's Manual* a number of new species have been validly described. These are also included in Table I.

2.4. Type Strains

A species usually consists of a series of strains from which one particular strain is designated as the type strain. The type strains of all bacterial species have been specified in the Approved Lists. In cases where a new species is described, the type strain has to be designated by the author at the time of publication of the name of that species.

The type strain is of great importance not only for nomenclatural purposes but also for classification at the species level since the species name is permanently associated with this strain. If a type strain is lost, a "neotype" strain will be selected which should resemble as closely as possible the description of the former type strain. To avoid the loss of type strains these have to be deposited with a culture collection, preferably with one of the recognized service culture collections.

The concept of the type strain has proven very useful. This strain is the name bearer and all strains belonging to a species should be highly related to its type strain. If, however, strains are recognized in subsequent studies as separate species, they must receive new names. The type strain and all remaining strains retain the original species name. Type strains are important for comparative work in diagnostic bacteriology.

The main problem with the concept of a type strain is that this strain is not

necessarily characteristic or typical for the species. This follows from the fact that a new species may be described only on the basis of one single isolate. This isolate would be automatically the type strain of the new species. If, later on, additional strains of this species can be isolated, it may be found that the type strain is rather untypical. To overcome this problem, species descriptions should be based ideally on the study of about 25, but certainly not less than 10, strains (Sneath, 1977). Then the most typical strain should be selected as the type strain.

3. CURRENT PROBLEMS IN *BACILLUS* CLASSIFICATION

3.1. Genus Definition

The use of spore formation as a main criterion to classify bacterial isolates with the genus *Bacillus* is complicated by the fact that sporulation often depends very much on the conditions under which a culture is tested. Many *Bacillus* species or strains sporulate poorly, if at all, on commercially available media or have been shown to sporulate only under more or less narrowly defined conditions. Gordon and Ryneerson (1963) and Gordon *et al.* (1973) observed that with most *Bacillus* strains sporulation is best on nutrient agar (e.g., Difco) where the water is replaced partly (10–50%) by soil extract. Our own experiments have shown that in most cases soil extract can be replaced by 10–50 mg/liter manganous sulfate. Sporulation generally requires higher concentrations of certain trace elements, especially manganous ions, than does vegetative growth (Charney *et al.*, 1951; Kalakoutskii and Dobritsa, 1984). Analysis of peptones and other ingredients of complex media have shown that these may contain only very small concentrations of such elements (Bovallius and Zacharias, 1971).

Another problem in classifying a nonsporulating strain with the genus *Bacillus* is the Gram reaction. In contradiction to most lecturers and their textbooks, species or strains of the genus may be found constantly as gram-negative or only in the very early stages of growth as gram-positive. It is obvious that a nonsporulating, gram-positive or gram-negative rod will usually be misclassified. For example, strains thought to belong to the genus *Lactobacillus* in fact have been identified as strains of the lactic acid-producing *B. coagulans*.

The genus *Bacillus* is now generally recognized to be more heterogeneous than other bacterial genera. This is apparent from the variety of carbohydrate metabolism and from differences in cell wall composition displayed by the individual species. From numerical taxonomy studies (Logan and Berkeley, 1981; Priest *et al.*, 1981) it was suggested that the genus may be best divided into three or more different genera. This is supported by studies on the DNA base composition of *Bacillus* strains, which is in the range of 32–69 mole% G+C. From theoretical aspects, species of the same genus should not differ more than 10–15% in their DNA base composition (DeLey, 1978). However, additional taxonomical studies are needed, especially comparisons of the rRNA cistrons by rRNA homology experiments or by 16S oligonucleotide catalogue similarities, to provide a sound basis for splitting the genus into several genera.

3.2. Species Concept

The basic group in bacterial taxonomy is the species, although the definition of a bacterial species has often given rise to discussions and arguments among microbiologists. Stanier *et al.* (1976) define a species as a group of strains that show a high degree of overall phenotypic similarity and that differ from related strain groups with respect to many independent characters.

Gordon (1978) states that a bacterial species is a concept represented by a group of strains from a variety of sources containing freshly isolated strains, stock strains maintained in laboratories for varying periods of time, and their variants, which have in common a set or pattern of correlating stable properties. Such properties separate the different groups of strains. In this connection it is noteworthy that freshly isolated *Bacillus* strains may change their properties during serial transfer (Allen, 1953). This may also be true for cultures which are routinely transferred only after a pasteurization step (Hayase *et al.*, 1974; Kadota *et al.*, 1978).

The presently recognized species concept in the genus *Bacillus* is mainly based on the views held by Gordon (1978) and on her practical studies (Gordon *et al.*, 1973). Since the resulting classification scheme is based on the study of approximately 50 properties, the question arises as to whether this number of characters is sufficient for an adequate characterization of a strain. Does it really allow us to define a species?

The number of possible experiments that can be performed for the characterization of bacteria is extremely large and in practice it would be impossible to perform all such experiments. Therefore,

the bacterial taxonomist can never be sure that he has performed the right experiments for taxonomic purposes; he may well have failed to perform certain experiments that would have shown him significant clustering in a collection of strains, and therefore erroneously conclude that he is dealing with a continuous series. There is no way to get around this difficulty, except to make phenotypic characterizations as exhaustive as possible. (Stanier *et al.*, 1976)

Such an exhaustive description of phenotypes is far from being achieved within the genus *Bacillus* today. The molecular weight of DNA in bacteria is around 1×10^9 to 8×10^9 , which is enough to specify some 1500–6000 average-sized genes. Therefore, a battery of about 50 tests would at most assay between 1 and 3% of the genetic potential of a *Bacillus* strain. Whereas a group of strains may be completely or nearly identical in this restricted number of phenotypic properties, its members may be totally different in the 97% of the properties not tested.

If such an assumption is right, one should expect that at least some of the presently recognized *Bacillus* species are not real taxospecies (which means a group of strains of high mutual similarity separated by a phenetic gap from nearby taxospecies) but only nomenspecies (which means species that carry binomial names whatever their taxonomic status is in other respects).

The first observations in this regard came from Gordon *et al.* (1973) themselves, who found that

the component strains of many species are relatively heterogenous and form a graduated series of differences like the graduation of a spectrum in which colors fade into one another in sequence. If a few strains that happen to come from the ends of such a spectrum-like species are examined, distinctive differences may

be evident; however, inclusion in the study of more strains will disclose large numbers of intermediate types that obliterate the demarcation noted at first.

A numerical analysis using the data provided by Gordon *et al.* (1973) clearly showed that within many *Bacillus* species different clusters of strains can be observed having only a rather low level of similarity (Priest *et al.*, 1981). Recent studies on the genetic relatedness of strains of several *Bacillus* species, based on DNA base composition and on DNA/DNA reassociation experiments, have confirmed the heterogeneity of many of the presently recognized *Bacillus* species. Such studies, first applied to problems in bacterial classification more than 20 years ago, have now also become very important in the taxonomy of the genus *Bacillus* (see Sections 4.4 and 4.5).

4. CRITERIA AND TECHNIQUES FOR *BACILLUS* CLASSIFICATION

4.1. Cultural and Morphological Characteristics

Earlier taxonomists, for lack of other suitable tests, laid strong emphasis on the form of surface colonies of *Bacillus* strains. Often small differences in colony structure, when compared to known strains, have been used to create new species. We now know, however, that colony appearance may vary considerably with such environmental factors as composition or moisture content of the medium or temperature or atmosphere of incubation. With the exception of strains consistently forming only small colonies, colony diameter depends largely on the number of colonies developing on a plate as well as on the concentration of nutrients or the amount of agar poured into a plate.

Whereas strains of a number of *Bacillus* species form rather characteristic colonies on a certain medium, strains of other species, like *B. subtilis* or *B. pumilus*, may show extreme variation. Certain *Bacillus* species tend to swarm or to migrate in the form of motile colonies over the whole plate or to grow over the plate in the form of rhizoid colonies. Again this may depend on cultural conditions. Even within a pure culture colonies showing different degrees of translucence or opaqueness may be observed. This is often due to different degrees of sporulation within the colonies. With such strains it may be rather difficult to decide whether a given culture can be considered as a pure culture.

In spite of the limitations mentioned above and the feeling of Cowan (1978) that colony form is one of the relics of nineteenth century bacteriology, it is nevertheless useful to describe the appearance of colonies of strains of new species under specific conditions since this may still be helpful in later identification work. The verbal descriptions may be difficult to choose but may be based on the vocabulary used by Salle (1973). A description of colonies of *Bacillus* species was given by Smith *et al.* (1952).

Cells of *Bacillus* strains occur singly or in chains which may be of considerable length. Filaments are often seen but in most cases they consist of chains whose cross walls are difficult to detect by ordinary microscopy.

Whereas the length of *Bacillus* cells may vary substantially due to different

physicochemical conditions of growth, the width of cells is a more constant feature. However, this may vary according to the presence or absence of carbohydrates in the medium. When grown on nutrient agar, only a few *Bacillus* species have a cell width $> 1 \mu\text{m}$. This is characteristic for *B. cereus*, *B. anthracis*, *B. mycoides*, *B. thuringiensis*, and *B. megaterium*.

On nutrient agar, *Bacillus* species form typical endospores which are best detected under phase contrast microscopy by their brightness. They may be cylindrical to oval or round. In a few cases kidney- or banana-shaped spores may be produced. Inclusion bodies containing poly- β -hydroxybutyrate, a storage product formed by many bacteria, may sometimes be misinterpreted as endospores.

The spore-bearing cell, the sporangium, is another characteristic and important feature of *Bacillus* species; during the formation of endospores, the sporangium may remain unchanged in shape or may swell. With the latter types, different sporangial shapes may develop depending on the location of the spore in the sporangium (central, paracentral, subterminal, terminal, lateral). Only exceptionally will swollen and unswollen sporangia be formed within the same culture (e.g., *B. coagulans*).

For studying the microscopic appearance of vegetative or spore-bearing cells, staining is not recommended. Cells are best observed on slides freshly coated with a thin layer (0.3–0.4 mm) of a 2% water agar (purified agar). After adding a small drop of a distinctly turbid cell suspension, the preparation is covered by a cover slip and is examined using a phase contrast microscope with an oil immersion objective.

Earlier bacteriologists spent much time describing the cell morphology of *Bacillus* strains. Today cell morphology is often considered as less important, and small but typical differences between species are not described or detected. Nevertheless, such specific differences do exist and may be helpful in identification work. The preparation of an atlas on the morphology of *Bacillus* species, based on strains known to belong to a certain species from DNA/DNA reassociation experiments, would be a worthwhile task.

4.2. Physiological and Biochemical Characteristics

It was mentioned above that one of the problems in classification is the adequate characterization of strains. This means not only what properties should be tested, but also which methods should be applied, since results may be strongly influenced by the method used. For example, reduction of nitrate to nitrite can be tested by KJ starch paper. However, this is a far less sensitive method for nitrite detection than a reagent composed of a sulfanilic acid, acetic acid, and dimethyl- α -naphthylamine.

The ability to grow anaerobically is sometimes tested in an anaerobic jar where oxygen is removed by a catalyst in the presence of hydrogen (and where oxygen may still be present in a concentration up to 1%!) or by applying the Hungate technique whereby oxygen is totally excluded. For the detection of acetoin (Voges–Proskauer test) several formulae are described, all of which may lead to different results depending on the strain under study. For several reasons it would be best to apply a common series of standardized tests to all bacteria. However, for historical reasons, different tests and methods are applied to different groups of bacteria.

As to the genus *Bacillus*, where the present classification is based on the studies of Smith *et al.* (1952) and Gordon *et al.* (1973), it is essential to apply the methods

and media used by these authors. Recently, these methods were extensively described (Claus and Berkeley, 1986). Use of other methods may give false-positive or false-negative results and lead simply to mischaracterization with respect to the presently recognized classification scheme.

4.3. Chemical Characteristics

The use of chemical characters and the chemical variation in bacteria have proven to be of value in bacterial taxonomy, and it seems likely that chemical properties will become an essential part in the description of genera and/or species.

4.3.1. Cell Wall Composition

The fundamental polymer common to the walls of most bacteria is the peptidoglycan murein. It is a heteropolymer made up of glycan strands crosslinked through short peptides (see Chapter 9). Glycan strands consist of *N*-acetylglucosamine and *N*-acetylmuramic acid, a lactyl ether of *N*-acetylglucosamine. The lactyl moiety provides a site to which a peptide chain can be attached. Adjacent peptide chains in the peptidoglycan may be crosslinked by peptide bonding.

Peptide chains always consist of alternating L- and D-amino acids and the most common sequence is L-alanine/D-glutamic acid/L-diamino acid (mostly *m*-diaminopimelic acid or L-lysine). Whereas in gram-negative bacteria the chemical composition and structure of the peptidoglycan is rather uniform and therefore generally of little value as a character for classification, gram-positive bacteria reveal a great variation in the composition and structure of the peptide chain, which may be crosslinked directly or via an interpeptide bridge. About 100 different peptidoglycan types have been described (Schleifer and Kandler, 1972; Schleifer and Seidl, 1985; Stackebrandt *et al.*, 1987). Since the structure and composition of the peptidoglycans formed by different bacteria are generally stable and do not depend on cultivation conditions, the cell wall composition in many gram-positive bacteria is often a very useful chemotaxonomic marker. Methods for studying the cell wall composition have been published by Schleifer (1985) and Schleifer and Seidl (1985).

Table II. Cell Wall Composition of Round Spore-Forming *Bacillus* Species^a

Species	Peptidoglycan type
" <i>B. aminovorans</i> "	<i>meso</i> -A ₂ pm-direct
<i>B. globisporus</i>	L-Lys-D-Glu
<i>B. insolitus</i>	L-Orn-D-Glu
<i>B. marinus</i>	L-Lys-D-Glu
<i>B. pasteurii</i>	L-Lys-Ala-D-Asp
<i>B. psychrophilus</i>	L-Lys-D-Glu
<i>B. sphaericus</i>	L-Lys-D-Asp

^aData from Stackebrandt *et al.* (1987).

The murein type of most *Bacillus* species is of the direct linked *meso*-di-aminopimelic acid (mDpm or mA₂pm) version. However, few exceptions are known (Table II), and it is interesting to note that except for "*B. aminovorans*" all species forming round endospores are included among these exceptions (see Section 3.1.1, Chapter 9).

4.3.2. Fatty Acids and Polar Lipids

The importance of the fatty acid composition and the distribution of polar lipids for the taxonomy of the genus *Bacillus* has not been fully established. Results have been reviewed by Kaneda (1977) and Minnikin and Goodfellow (1981).

According to Kaneda (1977), 19 *Bacillus* species can be allocated to six groups with respect to their fatty acid composition. All groups except one (thermophiles) contain major amounts of branched chain fatty acids. Up to now a large portion of unsaturated fatty acids has been detected only in the group of psychrophilic *Bacillus* species. A unique fatty acid pattern has been described for *B. acidocaldarius* whereby the main compounds are ω -cyclohexane fatty acids. The remaining groups differ in the amount of unsaturated fatty acids formed and in their predominant compounds (*iso*-C₁₅, *anteiso*-C₁₄, and *anteiso*-C₁₅).

4.3.3. Isoprenoid Quinones

Isoprenoid quinones play an important role in electron transport and oxidative phosphorylation. They are located in the cytoplasmic membrane of the cell. Whereas in other bacterial taxa menaquinones, demethylmenaquinones, and/or ubiquinones have been detected, the major isoprenoid in strains of the genus *Bacillus* is usually an unsaturated menaquinone with seven isoprenoid units (MK-7). Minor components include MK-2 to MK-11, depending on the species or strain (Collins and Jones, 1981).

For certain species MK-8 or MK-9 have been reported as the main components. This could not be verified (Kroppenstedt, unpublished). However, strains of the not yet validly described "*Bacillus aminovorans*" contain MK-6 as the main component.

4.4. DNA Base Composition

It is now generally accepted that the DNA base composition (mol% G+C) is a constant and therefore a taxonomically important characteristic of bacterial species. Strains belonging to one and the same species do not differ in DNA base composition by more than 1–2%. Species belonging to the same genus usually have a mol% G+C within a range not broader than 10%. Whereas closely related species have similar mol% G+C values, it is important to recognize that two organisms with similar DNA base compositions are not necessarily closely related since the deoxyribonucleotides in the DNA may not be in the same linear order.

A short overview on the implication of nucleic acid studies for bacterial taxonomy was published by Johnson (1985a), and methods for the determination of the DNA base composition were critically reviewed by Johnson (1985a) and Owen and Pitcher (1985).

It was already mentioned that the DNA base composition of species of the genus *Bacillus* has been found to be in the range of 32–69 mol% G+C, showing that the genus is genetically heterogeneous. From a phylogenetic point of view, this needs further consideration.

At the species level, *Bacillus* strains, considered to belong to a single species according to phenotypic classification, have been shown to differ in their DNA base compositions far more than expected (Priest *et al.*, 1981), indicating the genetic unrelatedness of such strains. For example, 20 strains identified by Smith *et al.* (1952) as *B. megaterium* were found to vary in their DNA base compositions between 35 and 41 mol% G+C. Based on additional phenotypic studies and DNA/DNA reassociation experiments, it has been proposed that strains of *B. megaterium* be allocated to at least four different species (Hunger and Claus, 1981). The most striking example, however, is *B. circulans* where a study of 123 strains showed a continuous spectrum in the DNA base composition from 37 to 61 mol% G+C (Nakamura and Swezey, 1983). Studies are now in progress to determine the DNA base composition of strains of other *Bacillus* species. These studies will almost certainly lead to the exclusion of strains from certain species and, after DNA/DNA reassociation studies and phenotypical characterization, to the description of a series of new species.

One of the main problems in evaluating earlier data on the DNA base composition of *Bacillus* strains is the fact that values published by different authors for the same strain sometimes differ considerably. Therefore, a comparative study of the type strains of most *Bacillus* species was undertaken by Fahmy *et al.* (1985). Table III includes their data together with recently published values for new species.

4.5. DNA Relatedness

The methods available for determining the extent of DNA relatedness were reviewed by Johnson (1985b) and Owen and Pitcher (1985). When a DNA sample is heated, hydrogen bonds holding together the two strands of DNA are broken. This leads to the production of single-stranded DNA which, upon rapid cooling, remains separated. Under certain conditions specific reassociation of complementary strands occur to form double-stranded DNA. Since specific pairings are between the base pairs adenine and thymine and between guanine and cytosine, the reassociation reaction depends on the linear arrangement of the bases along the nucleic acid strands.

The measurement of the extent to which single-stranded DNA fragments from one bacterial strain reassociate (or hybridize) with single-stranded DNA fragments from another strain can be used to determine nucleotide sequence similarity or DNA homology.

Experience has shown that DNA–DNA reassociation data are of main relevance only when investigating relationships within and between bacterial species. Generally, bacteria of the same species reveal DNA homology values above 70% (Johnson, 1973, 1985b), although the exact level below which organisms are considered to belong to a different species is arbitrary. DNA reassociation experiments remain to be applied to a number of taxonomic problems of *Bacillus*.

Within the genus *Bacillus*, DNA homology has been examined within a series of species (Priest, 1981) and some of them have been found to include two or more

Table III. DNA Base Composition of *Bacillus*-Type Strains

Type strains ^a	Mol% G + C		Type strains ^a	Mol% G + C	
	<i>T_m</i>	BD		<i>T_m</i>	BD
<i>Bacillus anthracis</i>	33.2		<i>Bacillus subtilis</i>	42.9	43.1
<i>Bacillus thuringiensis</i>	33.8	34.3	<i>Bacillus badius</i>	43.8	43.5
<i>Bacillus mycoides</i>	34.2	34.1	<i>Bacillus pulvifaciens</i> ^c		44.0
<i>Bacillus fastidiosus</i>	35.1	35.1	<i>Bacillus polymyxa</i>	44.3	45.6
<i>Bacillus circulans</i>	35.5	35.4	<i>Bacillus alvei</i>	44.6	46.2
<i>Bacillus cereus</i>	35.7	36.2	<i>Bacillus thermoglucosidasius</i> ^d	45.0	
<i>Bacillus insolitus</i>	35.9	36.1	<i>Bacillus licheniformis</i>	46.4	44.7
<i>Bacillus lentus</i>	36.3	36.4	<i>Bacillus coagulans</i>	47.1	44.5
<i>Bacillus pantothenicus</i>	36.9	36.8	<i>Bacillus brevis</i>	47.3	47.4
<i>Bacillus alcalophilus</i>	37.0	36.7	<i>Bacillus pabuli</i> ^e		49.0
<i>Bacillus megaterium</i>	37.3	37.6	<i>Bacillus lautus</i> ^e		51.0
<i>Bacillus sphaericus</i>	37.3	37.1	<i>Bacillus azotofixans</i> ^f	51.6	
<i>Bacillus marinus</i>	37.6	38.0	<i>Bacillus stearothermophilus</i>	51.9	51.5
<i>Bacillus lentimorbus</i>	37.7		<i>Bacillus macerans</i>	52.2	53.2
<i>Bacillus pasteurii</i>	38.5	38.4	<i>Bacillus amylolyticus</i> ^e		53.0
<i>Bacillus azotoformans</i> ^b		39.0	<i>Bacillus validus</i> ^e		54.0
<i>Bacillus macquariensis</i>	39.3	41.6	<i>Bacillus gordonae</i> ^g		54.6
<i>Bacillus psychrophilus</i>	39.7	40.5	<i>Bacillus thermoruber</i> ^h	57.0	
<i>Bacillus globisporus</i>	39.8	39.7	<i>Bacillus tusciae</i>	57.7	
<i>Bacillus laterosporus</i>	40.2	40.5	<i>Bacillus acidocaldarius</i>	60.3	62.3
<i>Bacillus popilliae</i>	41.3		<i>Bacillus schlegelii</i>	64.3	66.3
<i>Bacillus firmus</i>	41.4	40.7	<i>Bacillus larvae</i>	—	—
<i>Bacillus pumilus</i>	41.9	40.7			

^aData from Fahmy *et al.* (1985) and Claus and Berkeley (1986).^bData from Pichinoty *et al.* (1983).^cData from Nakamura (1984c).^dData from Suzuki *et al.* (1983).^eData from Nakamura (1984a).^fData from Seldin *et al.* (1984).^gData from Pichinoty *et al.* (1986).^hData from Manachini *et al.* (1985).ⁱData from Bonjour and Aragno (1984).

Table IV. Genetic Heterogeneity of Some *Bacillus* Species Revealed by DNA Studies

Species	No. of strains studied	Mol% G + C	DNA homology groups			Ref.
			No. of strains	% D1 ^a	% D2 ^b	
<i>B. agarexensis</i> ^c	16	44.4	1	21		Hunger and Claus, 1981
		44.4-44.5	2	<24	97	
		47.3-49.0	6	<32	80-100	
		50.8-51.7	4	>96	96-100	
		52.2-52.7	3	<34	97-100	
<i>B. circulans</i>	50	37.4-38.9	10	>63	63-100	Nakamura and Swezey, 1983
		45.6-46.2	3	<28	81-94	
		47.0-48.5	3	<10	98-100	
		47.3-49.1	3	<20	76-85	
		48.5-50.0	6	<16	78-100	
		47.8-50.2	6	<12	71-100	
		50.3-51.8	5	< 8	92-100	
		50.2-51.5	5	< 3	70-100	
		52.6-52.7	4	<15	89-100	
		53.3-54.4	5	<10	79-100	
		38.7-39.7	6	<18	77-84	
		45.2-46.9	8	>80	80-94	
<i>B. coagulans</i>	17					Blumenstock, 1984
<i>B. globisporus</i>	5	51.6-52.3	3	<30	69-84	Nakamura, 1984b
		42.8-44.1	3	>90	90-98	
			2	<30	25	
<i>B. megaterium</i>	20	34.2	1	42		Hunger and Claus, 1981
		37.0-38.1	11	>78	78-95	
		37.4-38.8	2	<33		
		40.1-40.9	6	<34	57-70	
<i>B. sphaericus</i>	49	36	4	>77	77-91	Krych <i>et al.</i> , 1980
		35	7	<20	79-88	
		35-36	12	<23	68-93	
		34-36	15	<23	76-88	
		35-37	8	<23	66-100	
		35	3	<25	74-80	
<i>B. subtilis</i>	26		11	>67	67-100	Seki <i>et al.</i> , 1975
			15	<36	66-92	

^a% DNA homology to the type or reference strain.^b% DNA homology within the different groups.

DNA homology groups with high intragroup but low intergroup relatedness (Table IV).

5. OUTLOOK

The views presented above have already shown that the taxonomy of the genus *Bacillus* is in motion. Within the next few years extended descriptions of species will be generated which are not based simply on their phenotypes. Species descriptions will generally be based on more stable characters including chemotaxonomy and new methods will be more widely applied in characterization studies, e.g., pyrolysis mass spectrometry (Shute *et al.*, 1988), electrophoretic whole-cell protein patterns (Kerstens and De Ley, 1980; Jackman, 1985) or enzyme patterns (Baptist *et al.*, 1978; Sharp *et al.*, 1980).

The main progress, however, will be gained by basing *Bacillus* classification on genetic relatedness. This will lead to a more unifying concept of a *Bacillus* species which will be subject to less radical changes. A recent report on reconciliation of approaches to bacterial systematics was made by Wayne *et al.* (1987).

It is expected that the genus will be split up into genera of closely related species and the position of such genera in a phylogenetic classification scheme will be elucidated.

An important aspect in these developments is diagnostic bacteriology. It is the task of any taxonomist in the *Bacillus* field not to neglect practical aspects and to give advice to manage the identification of new isolates by providing reliable identification schemes.

6. CULTURE COLLECTIONS

Research and development in bacteriology and biotechnology require that microbial strains described or mentioned in publications are available for independent study. It is thus necessary to keep and preserve reference cultures and other cultures of potential value for industry, medicine, agriculture, and other scientific activities. Culture collections provide this indispensable service to microbiologists by collecting and the long-term maintenance of well-characterized, authenticated strains of viruses, bacteria, fungi, protozoa, and, more recently, animal and plant cell lines.

Frantisek Kral (1846–1911) of Prague was the first to realize the need for supplying authentic cultures to the scientific community: he collected cultures which he made available for a fee to other workers. This collection was transferred to the University of Vienna in 1915 by Ernst Pribram. The next oldest collection, Centraalbureau voor Schimmelcultures (CBS), was founded in 1906 and still exists at Baarn, Netherlands.

Until the 1920s the main reason for the existence of collections was their value for taxonomic and epidemiological studies. In the 1930s, studies of microbial physiology and biochemistry entailed the preservation of microorganisms with special properties and productive qualities. Since then many collections have developed; the history of such collections can be read in Porter (1976). The recent role of

culture collections in microbiology and biotechnology and the services provided by them have been reviewed by Kirsop (1983a,b), Malik and Claus (1987), and Sly (1984).

Microbial collections are living libraries, a continuing reference source and an essential base for related research studies. In addition to the distribution of well-characterized, authenticated strains, culture collections are also centers of expertise in the fields of preservation, classification, and identification of microorganisms.

Biotechnologists may find culture collections helpful for screening, selection of certain strains, computer searches, patent deposit, training courses, consultancy, or contract research. In some countries several individual collections have founded associations such as UKFCC (United Kingdom Federation of Culture Collections), USFCC (United States Federation of Culture Collections), and JFCC (Japanese Federation of Culture Collections) to promote the interests of the associate collections, thereby making their stocks and services more widely known to the scientific community.

In Europe, ECCO (European Culture Collections' Organisation) was established in 1982 to ensure a more efficient service to biotechnology and industry by assisting collaboration between member collections and to avoid duplication of effort. Today 35 collections contribute to the joint activities of ECCO.

In recognition of the growing importance and continuance of the work of culture collections the WFCC (World Federation for Culture Collections) was founded as an Interdisciplinary Commission of the International Union of Biological Sciences and the International Union of Microbiological Sciences. The purpose of the WFCC is to promote activities that support the aims of culture collections. One of its main objectives is to attempt to find solutions, on an international basis, to the problems of distributing cultures of microorganisms that may arise through postal regulations, quarantine rules, patent laws, public health problems, and other factors of international importance. A special committee has been founded to help solve the problems of endangered collections. Another important task of WFCC is to organize training courses on modern methods of preservation and on the operation and management of culture collections. A worldwide network of data services will eventually be established, recording the location of, and information about, microorganisms maintained in culture collections. Derivative information will be published in the form of an updated *World Directory of Collections of Microorganisms* (Staines *et al.*, 1986) with lists of species maintained therein and related information.

A list of major collections around the world from which *Bacillus* strains may be obtained is given in Appendix 1. This list is by no means complete, with respect to both *Bacillus* in particular and culture collections in general. More detailed information can be drawn from Hawksworth (1985), Malik and Claus (1987), and Staines *et al.* (1986). Detailed information about fields of interest and services of these collections can be obtained from the collections listed herein or from the international organizations mentioned. In this context it would be appropriate to summarize the function of the international organizations, MSDN (Microbial Strain Data Network), MINE (Microbial Information Network Europe), and MiCIS (Microbial Culture Information Service). The objectives in the case of MSDN are to produce an electronic catalogue, in the form of a referral system, which indicates in which collection information about a particular strain can be found. MINE is developing a European computer network between collections, with the aim of compiling a mini-

mal data set for all the strains held by the collections. This information will be published in the form of a joint catalogue, and developed into a complete data bank in the not-too-distant future. MiCIS, which had been built up in England mainly for industrial users as a centralized data bank system, is founded on similar principles. Cooperation between these organizations beyond national borders is under discussion.

One service offered by a range of culture collections should be briefly mentioned here because of its particular importance to the development of biotechnology, namely, the deposition of microorganisms for patent purposes. The following text on this subject is drawn mainly from an information leaflet published by ECCO.

The rapid advances in genetic manipulation techniques and the allied boom in biotechnology over the past few years have led to an increased awareness of the enormous industrial potential of microorganisms. Large sums of money are being risked to exploit this potential and in the commercial development of new microbial processes. In such circumstances patent protection naturally becomes a major concern of any organization involved in modern industrial microbiology.

In addition to a written disclosure, the patent offices of most industrialized countries require the strain used in a microbiological invention to be deposited in a recognized public culture collection, whence it will eventually be available to the public.

Patent deposits are now regulated internationally by the Budapest Treaty (Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure), under which certain culture collections of a generally acknowledged high scientific standard are officially recognized as "International Depositary Authorities" (IDAs). A "Guide to the Deposit of Microorganisms under the Budapest Treaty" has been published by the World Intellectual Property Organization (1988).

The main advantages of the Budapest Treaty are first that no matter where and how many applications are filed, only one deposit need be made provided it is in a culture collection of IDA status. Second, the treaty has provided a uniform, internationally accepted system for depositing microorganisms in culture collections for patent purposes. Up to now, 22 states are party to the Budapest Treaty and there are presently 18 IDAs in the world, 14 of which are in Europe.

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Isolation and Identification of Aerobic Endospore-Forming Bacteria

3

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1. INTRODUCTION

The capacity of some bacteria to differentiate into heat-resistant endospores has traditionally been considered a taxonomic feature of crucial importance. Consequently, a diversity of organisms has been assigned to the family *Bacillaceae* ranging from the anaerobic gram-negative bacteria of the genus *Desulfotomaculum* to the aerobic gram-positive cocci of *Sporosarcina*. This review will concentrate on the ecology, isolation, and identification of the aerobic/facultatively anaerobic, gram-positive genera *Bacillus*, *Sporosarcina*, *Sporolactobacillus*, and *Thermoactinomyces*.

1.1. *Bacillus*

This genus contains the rod-shaped aerobic or facultatively anaerobic spore formers. A large genus, it currently comprises some 51 validly described species (see Chapter 2, Table I) and many additional species of uncertain taxonomic standing (Claus and Berkeley, 1986). The base composition of the DNA from *Bacillus* ranges from about 33 mol% G+C (*B. cereus*) to more than 60% for some extreme thermophiles, although most strains lie in the region 36–54% (reviewed by Priest, 1981; Fahmy *et al.*, 1985; see Chapter 2). This variation in chromosomal DNA composition is confirmed by 16S rRNA cataloguing, which has revealed that the genus *Bacillus* is a “deep” (evolutionarily ancient) group with equivalent evolutionary divergence to the combined families of *Enterobacteriaceae* and *Vibrionaceae* (Stackebrandt and Woese, 1981). It is therefore not surprising that a diversity of physiological and morphological types are contained in the genus. Three extensive numerical taxonomic studies have divided *Bacillus* into several taxa of approximate generic rank (Logan and Berkeley, 1981; Priest *et al.*, 1981, 1988) but detailed

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chemotaxonomic studies are needed before any firm decisions about splitting the genus are made.

Given the taxonomic heterogeneity of *Bacillus*, it is particularly impressive that the early attempts at classifying these bacteria and providing identification schemes were reasonably successful. Smith and his co-workers (1946, 1952) developed the use of spore morphology for division of the genus into three groups of strains: those containing oval spores that do not swell the sporangium, those with oval spores that distinctly swell the sporangium, and those with spherical spores. This scheme is still used routinely (see Section 4.1) but the approach has some drawbacks (Gordon, 1981), particularly for strains that sporulate poorly. Moreover, the current appreciation of the species diversity of *Bacillus* makes computerized probabilistic identification based on traditional phenotypic characters or chemosystematic features more appropriate (see Section 4.3).

1.2. *Sporosarcina*

Gram-positive cocci producing heat-resistant endospores were first isolated by Beijerinck in 1901 (see Norris, 1981, for review). These bacteria share some characteristics with the micrococci; in particular, the cell wall lacks diaminopimelic acid and contains principally alanine, glutamic acid, lysine, and glycine. But the low DNA base composition, around 40% (Claus, 1981), and rRNA cataloguing (Fox *et al.*, 1977) place *S. ureae* firmly within the family *Bacillaceae*. Recently, a second species was described, *S. halophila*, which has a distinctive cell wall, a DNA base composition of 40–41%, and shares only 30–36% DNA homology with *S. ureae* (Claus *et al.*, 1983).

1.3. *Sporolactobacillus*

The borderline between *Bacillus* and *Lactobacillus* is indistinct and both genera are included in the same “family” according to 16S rRNA cataloguing (Stackebrandt and Woese, 1981). Bacteria-sharing characteristics of both genera were isolated from chicken feed by Kitahara and Suzuki (1963). The lack of catalase, microaerophilic growth, and lactic acid fermentation likened these strains to *Lactobacillus*, but the production of typical endospores and cell walls containing diaminopimelic acid were consistent with inclusion in *Bacillus*. Accordingly, they were placed in a new genus, *Sporolactobacillus*, and other strains from the rhizosphere of wild plants, “*Sl. laevus*” and “*Sl. racemicus*,” have since been isolated.

1.4. *Thermoactinomyces*

Thermoactinomycetes are filamentous bacteria with a gram-positive branching mycelium which can be separated into substrate and aerial mycelia. The aerial mycelia are white/yellow and have the powdery appearance of typical, true actinomycetes but the aerial mycelium is often lost on repeated subculture. This genus differs from true actinomycetes by the production of refractile, heat-resistant endospores that contain dipicolinic acid (Cross *et al.*, 1968). The single spores are borne on both substrate and aerial hyphae. Cell wall structure (contains diamino-

pimelic acid; type III *sensu* Lechevalier and Lechevalier, 1970), DNA base composition, and 16S rRNA cataloguing are consistent with these bacteria being included as a genus in the *Bacillaceae*. Currently, five species are recognized: *T. vulgaris*, "*T. thalpophilus*," *T. dichotomicus*, *T. sacchari*, and the only mesophilic species, *T. peptonophilus*, but it is likely that more species will be described (Cross and Unsworth, 1981).

2. DISTRIBUTION AND ECOLOGY OF AEROBIC ENDOSPORE FORMERS

There is a scarcity of information on the distribution of aerobic endospore-forming bacteria in the environment. This is largely because the vast majority of these bacteria are not pathogenic for animals or plants and have therefore not attracted much interest or funding. Moreover, as pointed out by Slepecky and Leadbetter (1983), there is no single set of growth conditions which can be used to recover all known endospore-forming bacteria. Finally, it is relatively straightforward to identify the common bacilli using the schemes of Gordon *et al.* (1973), Gibson and Gordon (1974), and Parry *et al.* (1983), but many environmental isolates pose severe problems and are simply referred to as *Bacillus* species.

For the biotechnologist interested in novel microbial systems and products, it is important to be able to isolate bacteria with desired properties of temperature and pH tolerance, or simply novel bacteria. For this reason, the ecology of aerobic endospore-forming bacteria will be reviewed briefly so that suitable source material may be identified (see Table I).

2.1. Soil

The reservoir of *Bacillus* species is the soil, and strains have been isolated from the extremes of desert and Antarctic samples. Generally, soils of low organic content have a restricted flora dominated by *B. subtilis*, *B. licheniformis*, and *B. cereus*; however, with increasing fertility a wider range of species is encountered.

Numerical taxonomic studies have been particularly successful for the analysis of bacteria from soils and have contributed enormously to our understanding of the distribution of *Bacillus* species in various soils (reviewed by Goodfellow and Dickinson, 1985). When sampling the soil it should be remembered that, for example, thermophilic bacteria are not necessarily restricted to thermal sites nor alkaliphilic bacteria to high pH soils. A wide range of bacilli capable of growth at or above pH 10 can be isolated from normal soils (Aunstrup *et al.*, 1972; Horikoshi and Teruhiko, 1982) and *B. stearothermophilus* can be isolated from soils in temperate regions (Allen, 1953). Nevertheless, the highly thermophilic, acidophilic strains of *B. acidocaldarius* appear to be restricted to thermal springs and the surrounding soils (Darland and Brock, 1971; Loginova *et al.*, 1978), and it seems likely that certain haloalkaliphilic bacilli may be restricted to alkaline saline lakes (Weisser and Trüper, 1985). Although it is therefore advisable to sample exotic environments when searching for bacteria with particular characteristics, such as thermo- or pH tolerance, the frequency with which new species of *Bacillus* are isolated from normal soils suggests that many more taxa remain to be discovered given the correct enrich-

Table I. Sources and Common Habitats of Aerobic Endospore-Forming Bacteria^a

Organism	Reference strain isolated from	Common habitats (where known) and comments
<i>B. acidocaldarius</i>	Hot springs	Acid hot springs and soils, enrichment from neutral soils has failed
" <i>B. agar-exedens</i> "	Soil	—
<i>B. alcalophilus</i>	Human feces	Soil, water, dung
" <i>B. alcalophilus</i> subsp. <i>halodurans</i> "	Soil	Soil, water, sewage
<i>B. alvei</i>	Honeybee larvae suffering from European foulbrood	Soil; <i>B. alvei</i> is a saprophyte but common in bees with European foulbrood
" <i>B. aminovorans</i> "	Soil	—
<i>B. amyloliquefaciens</i>	Soil	Soil, industrial amylase fermentations
<i>B. amylolyticus</i>	Soil	—
" <i>B. aneurinolyticus</i> "	Human feces	—
<i>B. azotofixans</i>	Soil	Soil, rhizosphere of various grasses
<i>B. azotoformans</i>	Soil	Soil
" <i>B. apiarius</i> "	Dead larvae of honeybee	—
<i>B. badius</i>	Human feces	Dust, coastal waters, soil
" <i>B. benzeovorans</i> "	Soil	Soil
<i>B. brevis</i>	Soil	Foods, soil, seawater, and sediments
<i>B. cereus</i>	Soil	Soil; foods, especially dried foods, spices, and milk; seawater and sediments
<i>B. circulans</i>	Soil	Widespread in soil and decomposing vegetations; scarce in marine habitats
" <i>B. cirroflagellosus</i> "	Marine mud	—
<i>B. coagulans</i>	Evaporated milk	Beet sugar, canned foods, especially vegetables; medicated creams. Relatively scarce in soil
" <i>B. epiphytus</i> "	Marine phytoplankton	—
<i>B. fastidiosus</i>	Soil	Soil, poultry litter
<i>B. firmus</i>	Soil	Soil, seawater and marine sediments, salt marshes
" <i>B. freudenreichii</i> "	No reference strain	Soil, river water, and sewage
<i>B. globisporus</i>	Soil	Soil, mud, and water
<i>B. insolitus</i>	Soil	Soil, mud, water, and frozen foods
" <i>B. laevolacticus</i> "	Rhizosphere of ditch crow-foot	Rhizosphere of plants
<i>B. larvae</i>	Honeybee larvae suffering from American foulbrood	Infected brood and honeycombs. Presumably in soil around hives of bees

Table I. (Continued)

Organism	Reference strain isolated from	Common habitats (where known) and comments
<i>B. laterosporus</i>	Soil	Soil, water, dead honeybee larvae, rumen of animals
<i>B. lentimorbus</i>	Hemolymph of larvae of Japanese beetle	Causes milky disease of scarabaeid larvae. Presumably in soil inhabited by infected larvae
<i>B. lentus</i>	Soil	Seawater, marine sediments, salt marshes, and soil. Spices including black and red pepper
<i>B. licheniformis</i>	Soil	Soil; marine and freshwaters; foods, particularly dried foods, spices, and cocoa beans; compost; rumen of cattle. Cause of rope in bread
<i>B. macerans</i>	Unknown	Foods and vegetables; compost. Participates in retting of flax
<i>B. macquariensis</i>	Soil from Macquarie Island (sub-Antarctic)	Unknown
" <i>B. macroides</i> "	Cow dung	Decaying plant material
<i>B. marinus</i>	Seawater	Unknown
<i>B. megaterium</i>	Soil	Soils including desert soils; seawater and marine sediments; foods, particularly cocoa beans, dried foods, and spices
" <i>B. pacificus</i> "	Sand from seashore	Seawater
<i>B. pantothenicus</i>	Soil	Generally considered to be a soil inhabitant but also isolated from pharmaceutical products
<i>B. pasteurii</i>	Soil	Soil, water, sewage, urinals
<i>B. polymyxa</i>	Soil	Widely distributed in soil, decomposing plant matter and water
<i>B. popilliae</i>	Commercial spore dust	Causes milky disease of scarabaeid beetle larvae. Probably in soils inhabited by infected larvae
<i>B. psychrophilus</i>	Soil	Soil, water, mud; frozen foods, particularly vegetables
<i>B. psychrosaccharolyticus</i>	Soil	Soil, water, mud, and frozen vegetables
<i>B. pulvifaciens</i>	Dead larvae of honeybee	Unknown
<i>B. pumilus</i>	Soil	Ubiquitous in soil. Also found in seawater and marine sediments. Common in dried foods

(continued)

Table I. (Continued)

Organism	Reference strain isolated from	Common habitats (where known) and comments
" <i>B. racemilactius</i> "	Rhizosphere of wild lettuce	Rhizosphere of plants
<i>B. schlegelii</i>	Sediment of eutrophic lake	Unknown
<i>B. sphaericus</i>	Soil	Soil, marine and freshwater sediments, and foods
<i>B. stearothermophilus</i>	Unknown	Soil, foods including milk, canned foods, and sugar beet, dried foods
<i>B. subtilis</i>	Soil	Soil, marine and freshwaters and sediments; Foods including spices, cocoa, pulses, seeds, and bread
<i>B. thermoglucosidasius</i>	Soil	Unknown
" <i>B. thiaminolyticus</i> "	Human feces	Unknown
<i>B. thuringiensis</i>	Mediterranean flour moth	Pathogenic for lepidopteran larvae; common in soil
" <i>B. xerothermodurans</i> "	Soil	Unknown
<i>Sporolactobacillus inulinus</i>	Chicken feed	Rhizosphere of plants
<i>Sporosarcina ureae</i>	Soil	Soil, more common in urea-containing or well-manured soils
<i>S. halophila</i>	Salt marsh soils	Urinals
<i>Thermoactinomyces dichotomicus</i>	Unknown	Salt marsh soils
<i>T. peptonophilus</i>	Soil	Common in African soil samples; rare in British soils
<i>T. sacchari</i>	Sugar cane bagasse	Unknown
" <i>T. thalpophilus</i> " ^a	Manure	Soil
<i>T. vulgaris</i> ^a	Manure	Soil, cereals, moldy corn and compost; forced-air heating and humidification systems
		Soil, cereals, compost, and moldy hay; forced-air heating and humidification systems

^aNames are as proposed by Cross and Unsworth (1981). "*T. candidus*" is assumed to be a synonym of *T. vulgaris* (sensu Cross and Unsworth, 1981) and refers to nonamylolytic strains. *T. thalpophilus* is used to describe amylolytic strains.

ment and isolation conditions. Some recent examples include *B. thermoglucosidasius*, which produces extracellular α -glucosidase (Suzuki *et al.*, 1983), the denitrifier *B. azotoformans* (Pichinoty *et al.*, 1976), the nitrogen-fixing strains of *B. azotofixans* (Seldin *et al.*, 1984), the hydrocarbon-utilizing strains of *B. benzoovorans* (Pichinoty and Asselineau, 1984), and the H_2 chemolithotrophs of *B. schlegelii* (Schenk and Aragno, 1979).

With regard to spore formers other than *Bacillus*, *Sporosarcina ureae* is widely

distributed in soils; indeed, fertile soils may contain up to 10^3 sporosarcinae per gram (Gibson, 1935a). It seems likely that the primary habitat of this organism may be certain urban soils closely associated with man and dogs (Pregerson, 1973, quoted by Claus, 1981). *Sporosarcina halophila*, on the other hand, has only been isolated from coastal salt marsh soils (Claus *et al.*, 1983). Sporolactobacilli appear to be rare in the environment and are most commonly associated with soil (Doores and Westhoff, 1983), particularly the rhizosphere (see Section 2.4).

The availability of novobiocin as a powerful selection agent for most thermoactinomycetes simplifies ecological studies with these organisms. They seem to be widely distributed in soils, although they are more prevalent on plant materials (Cross, 1968). It should be remembered that not all thermoactinomycetes are resistant to 25 $\mu\text{g/ml}$ novobiocin, *T. peptonophilus* being a notable example (Nonomura and Ohara, 1971), and by the use of alternative selection procedures (see Section 3.2) other species may also be discovered. Of particular interest is the longevity of thermoactinomycete spores which have been recovered from archaeological sites dated from AD 85–95, providing strong evidence for the survival of spores for up to 1900 years (Unsworth *et al.*, 1977).

2.2. Water

2.2.1. Marine

Bacillus strains may constitute up to 20% of the total heterotrophic flora in seawater and, although even higher figures have been quoted, the average population seems to be around 9%. Coastal waters often have higher numbers of bacilli which tend to increase with temperature and depth but decrease with distance from the coast (see Bonde, 1981, for review). This presumably reflects "runoff" of soil bacteria into the sea. The likely terrestrial origin of these bacteria is emphasized by the isolation of thermoactinomycetes from streams, estuaries, coastal and marine locations (Cross, 1981; Attwell and Colwell, 1984). Seawater seems to be dominated by *B. licheniformis* followed by *B. subtilis* and by *B. pumilus* in nonpolluted samples. Other species encountered in low numbers include *B. brevis*, *B. firmus*, and *B. sphaericus*, largely in nonpolluted areas. Marine sediments generally contain a wider range of bacilli than the water column and may account for between 14 and 80% of the total number of heterotrophic bacteria. Again the frequency is influenced by distance from the coast and level of pollution, but *B. subtilis* and *B. pumilus* dominate coastal polluted sites and nonpolluted sites also contain *B. cereus*. Oceanic sediments contain a diversity of bacilli and, in addition to those noted above, *B. firmus* and *B. licheniformis* have been isolated (Bonde, 1981). In a numerical study of North Sea sediments, Boeyé and Herts (1976) found that *B. subtilis*, *B. licheniformis*, and *B. firmus* strains predominated.

It seems that most marine strains of *Bacillus* conform to established species and have no special characteristics. However, some species may be restricted to this environment, in particular organisms closely related to *B. firmus* and classified as "M" strains by Bonde (1981). Other marine bacteria such as "*B. epiphytus*" "*B. filicolonicus*," and "*B. cirroflagellosus*" (Zobell and Upham, 1944) are of uncertain taxonomic position.

Isolates from salt marshes and estuarine muds pose a taxonomic problem at present. These bacteria are often pigmented orange, yellow, or pink and are probably variants of *B. firmus* and *B. lentus* (Turner and Jervis, 1968; Gordon *et al.*, 1977). Although they have been described as a "spectrum" of strains, recent numerical taxonomic studies suggest they may constitute several defined taxa related to *B. firmus* (Logan and Berkeley, 1981; Priest *et al.*, 1988).

2.2.2. Freshwater

Most bacilli in freshwater probably represent bacteria of soil origin, the vast majority of organisms being gram-negative (Austin and Allen-Austin, 1985). *Bacillus* sp. are more common in sediments and include typical soil organisms such as *B. megaterium*, *B. cereus*, *B. firmus*, and *B. pumilus* (Allen *et al.*, 1983).

Of particular interest are the thermophilic bacilli isolated from hot springs and other freshwater thermal environments. These include *B. acidocaldarius* and the more commonly encountered *B. stearothermophilus*, "*B. thermodenitrificans*," *B. coagulans*, and "*B. thermocatenuatus*" (Golovacheva *et al.*, 1975, reviewed by Wolf and Sharp, 1981).

2.3. Animals

With the exception of the anthrax bacillus, the ecology of which has been comprehensively reviewed by Norris *et al.* (1981), bacilli are not highly pathogenic to mammals. *Bacillus cereus* has been associated with a range of opportunistic infections, notably bovine mastitis; abscess formation; septicemia; endocarditis; and ear, eye, and wound infections (Gilbert *et al.*, 1981; Norris *et al.*, 1981). The most prevalent illness from *B. cereus* is food poisoning associated with eating foods, particularly rice, in which toxigenic strains of *B. cereus* have been allowed to multiply. Two toxins, an emetic and a diarrheal toxin, are secreted by *B. cereus* strains and are responsible for the symptoms.

The diarrheal type of intoxication seems to be associated with contaminated meat, vegetables, puddings, and sauces while the vomiting type appears to be largely restricted to rice dishes. Using an API system it is possible to distinguish between emetic and other strains of *B. cereus* (Logan *et al.*, 1979). *Bacillus subtilis* and *B. licheniformis* have also been implicated in food poisoning and recovered in large numbers from patients' feces but pathogenicity has seldom been conclusively established (see Gilbert *et al.*, 1981, for review).

Various bacilli have been isolated from the feces of healthy individuals and animals (Table I) but these organisms largely represent transient inhabitants of the gut taken in with food. It is interesting to note at this point that various large organisms that produce endospores occur in the alimentary tract of animals. Many of these have two or more spores per cell; some grow as septate filaments and others are spirals. The taxonomy of these bacteria is unknown, nor is it clear whether they produce typical endospores containing dipicolinic acid (see Gibson and Gordon, 1974).

Bacilli are probably important members of the rumen ecosystem. Rumen isolates from cows, sheep, and goats identified as *B. licheniformis*, *B. circulans*, *B. coag-*

ulans, *B. laterosporus*, and unidentified facultative anaerobes resembling *B. pumilus* have been shown to hydrolyze hemicellulose and may thus assist in the nutrition of the host (Williams and Withers, 1981, 1983). Moreover, *B. macerans* has been implicated in ruminal nitrogen fixation (Jones and Thomas, 1974).

Insects

Bacilli from several species are pathogenic for a variety of insects. *Bacillus popilliae* and the closely related *B. lentimorbus* are obligate pathogens of various members of the family Scarabaeidae (Coleoptera). Since these bacteria sporulate in the hemolymph of living scarabaeids, they are generally isolated from infected larvae or the soil from infected areas (Bulla *et al.*, 1978; Milner, 1981).

Bacillus thuringiensis produces an intracellular crystal protein during sporulation that is toxic to various insect larvae. Different strains have different spectra of activity and some (e.g., var. *israelensis*) are pathogenic to larvae of mosquitoes and blackfly and others (var. *tenebrionis* and var. *San diego*) to some coleoptera including Colorado potato beetle and boll weevil (Hernstadt *et al.*, 1986). These bacteria are widely distributed in the soil and are generally isolated from infected insects.

Recently, some strains of *B. sphaericus* have been shown to be pathogenic to mosquito and blackfly larvae (see Chapter 11). *Bacillus larvae* causes American foulbrood of honey bees and can be isolated from infected animals and their environs. Other strains of *Bacillus* associated with bees, but apparently nonpathogenic, include *B. alvei*, "*B. apiarius*," *B. laterosporus*, and *B. pulvifaciens* (Bucher, 1981). *Bacillus circulans* has been associated with several insects but in general the bacilli, and particularly the aerobic species such as *B. megaterium*, *B. subtilis*, and so on, are uncommon in insects (Bucher, 1981; Breznak, 1982).

2.4. Plants

Bacillus species are not generally associated with leaves of plants which are largely colonized by gram-negative bacteria. Nevertheless, they have occasionally been isolated from olive tree leaves (Ercolani, 1978) and are commonly found in leaf litter (Goodfellow *et al.*, 1976). *Bacillus* seems also to be associated with tobacco leaves and may be important for the satisfactory fermentation of cigar leaf tobacco (reviewed by Norris *et al.*, 1981).

The rhizosphere and rhizoplane, on the other hand, contain large *Bacillus* populations. Organisms with more fastidious growth requirements including *B. macerans*, *B. polymyxa*, and sporolactobacilli are often isolated from root areas where they grow at the expense of root exudates. Conversely, some bacilli fix nitrogen and may provide the plant with nitrate in nitrogen-deficient soils. This is a subject of some controversy but strains of *B. polymyxa* and *B. macerans* from Welsh sand dunes may be associated with the ability of Marram grass to populate this nitrogen-poor habitat (Rhodes-Roberts, 1981). More recently, strains of the nitrogen-fixing species *B. azotofixans* were isolated from the rhizoplane of various plants (Seldin *et al.*, 1984).

Straw and cereals are heavily colonized with *Bacillus* and *Thermoactinomyces* spores, presumably of soil origin. In the field these may approach 10^5 per gram

representing the common soil *Bacillus* strains (Flannigan, 1987). Thermoactinomycete spores are particularly important in this respect because of their involvement in various hypersensitive reactions, although it should be remembered that other bacteria such as micropolysporas and saccharomonosporas are also involved (Kurup, 1984). *Thermoactinomycete* spores are also prevalent in mushroom compost (Kleyn *et al.*, 1981). Cereals such as rice and pulses have a varied *Bacillus* flora including *B. cereus* (Gilbert and Parry, 1977; Blakey and Priest, 1980).

2.5. Foods

Foodstuffs provide a useful source material for the isolation of novel strains of *Bacillus*. The *Bacillus* flora of foods is related to the distribution of these bacteria in soil, water, and plants and the resistance of the spore to heat and desiccation. Hence they tend to be more common in plant foods than those of animal origin and those that have been heated or desiccated generally possess an enriched and varied flora of bacterial spores. Because it is unrealistic to cover all foodstuffs from which bacilli have been isolated, this chapter concentrates on those foods in which bacilli are common.

2.5.1. Cocoa

To produce cocoa, the beans are fermented in their pods which contain a sterile sugary pulp. During this fermentation bacilli, originating from the soil and air, become the dominant organisms (about 10^6 per gram) and the spores survive the subsequent drying, roasting, grinding, and pressing involved in the manufacturing process. The usual flora of the cocoa fermentation includes *B. cereus*, *B. megaterium*, *B. subtilis*, *B. coagulans*, *B. pumilus*, and *B. stearothermophilus* and it is likely that other, currently unidentifiable bacteria are present (Ostovar and Keeney, 1973; Carr, 1981). Prior to roasting, the beans contain a variety of microorganisms but bacilli usually constitute 90% of the population which varies between 5×10^5 to 5×10^7 per gram. After roasting (40 min at 150°C), the population is reduced to about 10% and in one study comprised mainly *B. coagulans*, *B. stearothermophilus*, *B. licheniformis*, *B. megaterium*, and *B. subtilis* (Ostovar and Keeney, 1973). Cocoa powder that has been hygienically handled generally contains 10^2 – 10^4 *Bacillus* per gram (reviewed by Pivnick, 1980b).

2.5.2. Sugar

Sugar cane is essentially devoid of aerobic spore formers but beet sugar has a *Bacillus* flora derived from the soil. Sugar is extracted from the beet in a hot water diffuser which generally has a high population of facultative anaerobic thermophiles, mostly bacilli (Klaushofer *et al.*, 1971). In one of the most comprehensive studies of these bacteria, Klaushofer and Hollaus (1970) characterized some 80 isolates using numerical taxonomy. They defined four major groups: (1) *B. stearothermophilus* strains that are inert toward starch and casein; (2) *B. stearothermophilus* strains that are physiologically more active and have been termed "*B. thermodenitrificans*"; (3) *B. stearothermophilus*, characteristic of the reference strain; and

(4) *B. coagulans*. Obviously, this environment contains a diversity of thermophilic bacilli.

2.5.3. Spices

Dry spices contain those bacteria indigenous to the plant, soil, and air from where they originated. Fecal contamination by birds and insects is also a consideration. The drying process enriches for *Bacillus* spores, which are usually the dominant proportion of the population. *B. subtilis*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. firmus*, and *B. brevis* are commonly isolated (Goto *et al.*, 1971). Thermophilic bacilli and *B. cereus* are also encountered (Seenappa and Kempton, 1981). Generally bacterial counts are from 10^4 to 10^7 per gram except for cloves, mustard, and nutmeg, in which counts are lower (reviewed by Pivnick, 1980a).

2.5.4. Milk

The ubiquitous distribution of *Bacillus* makes them prominent milk spoilage organisms. Lecithinase-positive strains of *B. cereus* are responsible for the "broken" or "bitty" cream condition but other *Bacillus* occur in raw and pasteurized milk, including *B. subtilis*, *B. sphaericus*, *B. megaterium*, *B. pumilus*, and *B. firmus* (reviewed by Norris *et al.*, 1981). Thermophilic and thermotolerant bacteria including *B. stearothermophilus*, *B. licheniformis*, and *B. coagulans* have been isolated from pasteurized and dried skim milk (Chopra and Morthur, 1984). Psychrotrophic bacilli in milk have been described by Shehata and Collins (1971).

2.5.5. Frozen Foods

Frozen and chilled foods are a rich source of psychrophilic and psychrotrophic bacilli. Gyllenberg and Laine (1970) isolated 78 psychrophilic spore formers from refrigerated meats, fish, vegetables, and fruits and in a subsequent numerical taxonomic study confirmed the presence of *B. psychrosaccharolyticus*, *B. insolitus*, and a joint cluster representing *B. globisporus* and *B. psychrophilus*. Similarly, Sheard and Priest (198) recovered *B. insolitus*, *B. psychrophilus*, *B. psychrosaccharolyticus*, and unidentified psychrotrophic bacilli from frozen foods (reviewed by Priest and Barbour, 1985).

In conclusion, when searching for novel isolates of *Bacillus* it should be remembered that soil is not the exclusive habitat of these bacteria and by sampling more varied environments the "right" organism might be more readily isolated.

3. ISOLATION

Bacteria such as *B. subtilis* or *B. licheniformis* are robust, grow readily in most media, and can be easily isolated by the inexperienced microbiologist. In this section I shall therefore emphasize methods for the isolation of the rarer taxa of aerobic endospore-forming bacteria. The approach is based largely on the general strat-

egies for the isolation of novel microbes devised by Williams and his colleagues (Williams and Wellington, 1982; Williams *et al.*, 1984).

3.1. Enrichment of Samples

By sampling some of the more exotic habitats mentioned in Section 2, a preliminary enrichment of a particular group can be obtained. Thus diseased insects are likely to harbor large numbers of insect pathogenic bacteria and salt lakes will have a predominant flora of halophiles. Nevertheless, many "exotic" samples will contain high numbers of rapidly growing bacteria such as *B. cereus* that will overgrow the rarer and often less vigorous bacteria that are to be isolated. It is here that enrichment of the sample, either by inhibition of the unwanted organisms or selective growth of the desired microbe, is often the key to successful isolation.

For endospore-forming bacteria, the most common pretreatment is pasteurization of the sample at 80°C for 10 min or some similar procedure to eradicate vegetative cells. This invaluable enrichment step has some shortcomings as emphasized by Norris *et al.*, (1981): (1) only spore formers present as spores are isolated; (2) some spores are poorly heat-resistant, particularly those of psychrophiles (Michels and Visser, 1976), and could be destroyed; (3) suitable germination conditions may not be provided in the following growth phase. Thus heat treatment should not necessarily be adopted as a universal procedure for enrichment of *Bacillus* and related taxa, and sometimes ethanol treatment (Bond and Favero, 1977), use of some other solvent, or UV light irradiation may be a more appropriate spore selection agent. Differences in germination rate can be exploited at this stage to provide additional selection. Spores of many of the more common species such as *B. subtilis* and *B. cereus* germinate rapidly in simple media but spores of the less vigorous species do not. By incubating a heat-treated sample in a suitable medium for several hours and repeating the heat treatment, *B. subtilis* and similar organisms can be destroyed and spores of *B. macerans*, *B. polymyxa*, and other (rarer species) often remain. The use of selective agents to inhibit germination offers a powerful approach to the isolation of novel *Bacillus* strains that, to the author's knowledge, has not been exploited.

Enrichment is generally viewed as increasing the proportional numbers of a particular type of organism in a sample by providing a selective environment for the growth of the desired organism. Both nutritional and physical constraints can be used (for review, see Williams *et al.*, 1984). In theory, an enrichment procedure developed for any type of microbe can be made specific for *Bacillus* by first pasteurizing the sample material. In this way a variety of unusual strains of *Bacillus* have been isolated (Table II).

Nutritional enrichments include the selective isolation of *B. benzoovorans*, a hydrocarbon-utilizing organism, from pasteurized soil by providing benzoate as the sole carbon and energy source in a minimal medium. The organism normally grows as flexible immotile filaments (trichomes) enclosed in a sheath, but vigorously shaken cultures grow as typical motile rods (Pichinoty and Asselineau, 1984). Enrichment seems to be a prerequisite for the isolation of hydrocarbon-utilizing *Bacillus* since the great majority of species do not grow or grow only weakly on these substrates (Kacholz and Rehm, 1977). Other carbon sources that have been used to isolate specific *Bacillus* types include agar, chitin, and various other polysaccharides.

Table II. Some Enrichment Systems for the Selective Isolation of Bacilli

Organism	Isolation procedures	References
<i>B. acidocaldarius</i>	Medium pH 4 incubated at 60°C	Darland and Brock, 1971
" <i>B. agar-exdents</i> "	Minimal medium with yeast extract, biotin, and 0.1% agar pH 7.9–8.2	Hunger and Claus, 1978
<i>B. alcalophilus</i>	Direct plating on nutrient agar pH 8.5–11	Horikosi and Teruhiko, 1982
" <i>B. aminovorans</i> "	Trimethylamine as sole N ₂ and C source	Claus and Berkeley, 1986
<i>B. azotofixans</i>	N ₂ -free thiamine biotin agar. Anaerobic incubation	Seldin <i>et al.</i> , 1984
<i>B. azotoformans</i>	Peptone medium in an atmosphere of N ₂ O	Pichinoty <i>et al.</i> , 1976
<i>B. benzoevorans</i>	Minimal medium with benzoate as carbon and energy source	Pichinoty and Asselineau, 1984
<i>B. coagulans</i>	Enrichment in nutrient broth plus glucose at pH 5.5 and 50°C. Incubate plates of the same medium anaerobically at 50°C	Allen, 1953
<i>B. fastidiosus</i>	Minimal medium with uric acid or allantoin as sole source of C and N ₂	Bongaerts and Vogels, 1976
<i>B. firmus</i>	Marine or seawater agar incubated at 22°C for 7 days	Fahmy, 1978 (quoted in Claus and Berkeley, 1986)
<i>B. licheniformis</i>	Anaerobic enrichment in peptone nitrate medium	Claus, 1965
<i>B. megaterium</i>	Glucose minimal medium with nitrate as sole N ₂ source	Claus, 1965
<i>B. pantothenicus</i>	Enrich in nutrient broth plus 4% NaCl at 37°C	Proom and Night, 1950
<i>B. pasteurii</i>	Medium containing yeast extract, (NH ₄)SO ₄ , and 5% urea, pH 9.0. Aerobic	Willey and Stokes, 1962
<i>B. polymyxa</i>	Peptone starch medium in tubes with Durham's tubes. Gas formation is presumptive	Ledinham <i>et al.</i> , 1945
<i>B. sphaericus</i>	Medium containing arginine as sole carbon and nitrogen source and streptomycin is selective for insect pathogens	Yousten <i>et al.</i> , 1985
<i>B. stearothermophilus</i>	Various media at 65°C	Allen, 1953
<i>S. ureae</i>	Urea (1%) in tryptic soy yeast agar pH 8.5	Claus, 1981
<i>Thermoactinomyces</i>	Various media containing novobiocin (25 µg/ml) incubated at 50°C	Hobbs and Cross, 1983

It should be noted, however, that enzymes responsible for the hydrolysis of these macromolecules are extracellular and normal enrichment procedures work poorly in this context since most of the organisms in the culture will be able to grow on the degradation products and enrichment is not selective (for review, see Priest, 1983). *Bacillus fastidiosus* is an unusual organism that utilizes uric acid as the only source of energy, carbon, and nitrogen. Selective enrichment of *B. sphaericus* has been achieved by providing acetate as a sole carbon source (Massie *et al.*, 1985).

Nitrogen nutrition has also been exploited for the enrichment of unusual bacilli. Urea-decomposing bacilli are common in soil (Gibson, 1935b). *Bacillus pasteurii* and *Sporosarcina urea* can be isolated from pasteurized soil following enrichment in a liquid medium comprising yeast extract and urea at pH 9.0 and incubated at 30°C, although in most cases enrichment is unnecessary. Nitrogen-fixing strains of *Bacillus*, including the recently described *B. azotofixans*, can be enriched from soil by anaerobic culture in a nitrogen-deficient medium supplemented with vitamins (Seldin *et al.*, 1984) or yeast extract (Rhodes-Roberts, 1981). *Bacillus* able to use N₂O as a terminal electron acceptor were isolated by Pichinoty *et al.* (1978) after enrichment of pasteurized soil in media containing peptone, incubated at 32°C in an atmosphere of N₂O. These bacteria were assigned to the species *B. azotoformans* (Pichinoty *et al.*, 1978). Other novel, denitrifying *Bacillus* strains have been isolated using nitrite for enrichment (Pichinoty *et al.*, 1979).

Facultative lithotrophic bacilli (*B. schlegelii*) able to grow with H₂ and CO₂ were isolated from surface layers of sediments from a eutrophic lake following enrichment in minimal medium at 65°C in an atmosphere of O₂, CO₂, and H₂ (Aragno, 1978). Carbon monoxide-oxidizing *Bacillus* strains have also been isolated using similar procedures (Meyer and Schlegel, 1983).

Other chemical factors that can be used to enrich for specific strains of *Bacillus* include pH. Low pH combined with high temperature was used to enrich for *B. acidocaldarius* (Darland and Brock, 1971), and a few other highly acidophilic bacilli have been described from soil (Hippchen *et al.*, 1981).

Sporolactobacillus can be enriched in MRS broth containing α -methyl glucoside, potassium sorbate, and bromocresol green indicator adjusted to pH 5.5 with acetic acid. It is incubated at 37°C for at least 7 days and lactobacilli are removed by heat treatment prior to plating onto solid media (Doores and Westhoff, 1983). Glucose yeast extract peptone medium (Kitahara and Suzuki, 1963) was originally used for sporolactobacilli but interference from clostridia was a problem.

High pH is selective for *B. alcalophilus*, other alkaliphilic bacilli, and alkaliphilic variants of neutrophilic bacilli. Enrichment at high pH with 12% NaCl resulted in the isolation of "*B. alcalophilus* subsp. *halodurans*" (Boyer *et al.*, 1973). Other halotolerant species include *B. pantothenicus*, which might be isolated following enrichment in NaCl.

The principal physical factor used for enrichment purposes is temperature. Most *Bacillus* thermophiles grow at 55–65°C but the lower temperature is unsuitable for enrichments since many mesophilic bacilli, such as *B. licheniformis* or *B. brevis*, will grow at this temperature (Gordon *et al.*, 1973). Higher temperatures (70–75°C) were used by Heinen and Heinen (1972) to isolate the thermophiles "*B. caldodenax*," "*B. caldolyticus*," and "*B. caldevelox*."

Psychrophilic bacilli were originally isolated by Larkin and Stokes (1966) from

pasteurized soil, water, and mud following enrichment in tryptone soy broth at 0°C for 2 weeks and Laine (1970) successfully employed a temperature of 2°C for 2 weeks. Again, for maximum selectivity it is important to regulate the temperature carefully since many mesophilic bacilli will grow at 5°C including *B. megaterium*, some strains of *B. subtilis*, and some strains of *B. circulans* (Gordon *et al.*, 1973).

3.2. Growth in Laboratory Media

The next stage in the isolation of microbes is growth on isolation media. All media are to some extent selective and thus many of the opportunities of enrichment apply here also. Indeed in many instances enrichment will not be necessary and highly selective media can be used to isolate specific taxa directly from the sample material, particularly if the organisms are present in high numbers.

The majority of bacilli grow well on commercial nutrient agar or tryptone soy agar, although it must be varied according to pH and salt concentration for various physiological types. Spizizen salts (see Appendix 2) is a useful minimal medium and soil extract agar (Gordon *et al.*, 1973) is valuable for promoting sporulation in strains that do not sporulate readily. The highly fastidious insect pathogenic bacteria (*B. popilliae*, *B. larvae*, and *B. lentimorbus*) are generally grown on J-agar, a tryptone, yeast extract, and glucose medium (Gorden *et al.*, 1973).

Selective media have been developed for some taxa, particularly those of medical or commercial importance. Selective media for *B. anthracis* have been reviewed (Norris *et al.*, 1981; Parry *et al.*, 1983) and will not be covered here. A selective/indicator medium was devised for *B. cereus* by Mossel *et al.* (1967) and exploits the characteristic lecithinase activity and inability of these bacteria to ferment mannitol. Selectivity is provided by inclusion of polymyxin. Similarly, Kim and Goepfert (1971) described an egg yolk-polymyxin medium and Kendall's BC medium is based on mannitol fermentation and the egg yolk reaction (Parry *et al.*, 1983).

A selective medium (BATS) for strains of *B. sphaericus* that are pathogenic for mosquitos has been developed by Yousten *et al.* (1985). Although the addition of streptomycin to a complex medium is useful for *B. sphaericus* (Hertline *et al.*, 1979), BATS medium which contains arginine as sole nitrogen and carbon source and streptomycin is specific for the insect pathogenic strains. Few other selective or indicator media have been designed for specific *Bacillus* species. Suitable media for some specific groups are given in Table II.

Thermoactinomycetes can be selectively isolated by plating pasteurized samples onto Czapek, yeast extract casamino acids (CYC) agar (Hobbs and Cross, 1983) containing 25–100 µg/ml novobiocin and incubated at 55°C. Nevertheless, *T. peptonophilus* is sensitive to novobiocin as may be other undiscovered species. An alternative procedure is to seed the pasteurized sample onto the surface of a nitro-cellulose filter placed on a CPC agar plate. After incubation for 1–2 days the membrane is removed, and only mycelia that have penetrated through the membrane and colonized the surface of the agar will grow on subsequent incubation.

It seems clear that the diversity of known endospore formers is limited only by the ingenuity of the investigator and, as more and varied enrichment procedures and selective media are developed, so it is likely that new and novel taxa will be discovered. General strategies for the isolation of novel bacteria using numerical

Table III. Example of Output from the DIACHAR Program for the Listing of the Most Diagnostic Character States of Each Taxon

Taxon character	<i>B. pantothenicus</i> character state	Score
Growth 10% NaCl	Positive	0.781
Growth 50°C	Positive	0.774
Spores terminal	Positive	0.759
Nalidixic acid	Positive	0.721
Anaerobic growth	Positive	0.716
Mannitol fermentation	Negative	0.707

taxonomic data bases have been devised by Williams *et al.* (1984). Since it is anticipated that the application of these techniques to *Bacillus* should be particularly valuable, they are described briefly below.

3.3. General Strategies for the Isolation of Novel Bacteria

An end product of most numerical taxonomic studies is a frequency table indicating the percentage occurrences of positive feature for the clusters or taxa obtained. The exploitation of these data bases for the design of selective isolation conditions for rare or unusual taxa has been very successful (Williams and Wellington, 1982; Wellington and Cross, 1983; Goodfellow and Williams, 1986). Comprehensive data bases, such as those for *Streptomyces* (Williams *et al.*, 1983) and *Bacillus* (Priest *et al.*, 1988), contain information on antibiotic or extracellular enzyme synthesis by bacteria from a wide variety of taxa. These frequency tables also indicate sugar fermentation patterns, temperature and pH growth profiles, antibiotic resistance patterns, and other useful physiological data. Having highlighted a particular taxon for the synthesis of a product of interest, scrutiny of the frequency table usually reveals a set of conditions that would favor the desired organisms but few others. Thus by combining relevant carbon and nitrogen sources, pH, and antibiotics, highly selective media can be formulated. To assist this process the DIACHAR program (Sneath 1980a) highlights the most diagnostic features of a taxon. In the example shown in Table III, a combination of 10% NaCl, nalidixic acid, and high temperature of incubation should be highly selective for the isolation of *B. pantothenicus*. This approach has been very successful for the isolation of antibiotic-producing actinomycetes (Goodfellow and Williams, 1986).

4. IDENTIFICATION

The major genera of aerobic endospore-forming bacteria can be readily distinguished from their morphology and some simple physiological tests (Table IV). Identification to species level is, however, more problematic. Identification of

Table IV. Differential Characteristics of the Genera of Aerobic, Endospore-Forming Bacteria^a

Characteristic	<i>Bacillus</i>	<i>Sporolactobacillus</i>	<i>Sporosarcina</i>	<i>Thermoactinomyces</i>
Morphology	Rods	Rods	Cocci	Mycelial
Facultative anaerobes or microaerophiles	d	+	—	—
Catalase	+	—	+	+
Mol% G + C	32–69	38–40	40–42	52–55

^a+, 90% or more of strains positive; —, 10% or less of strains positive; d, substantial proportion of species differ.

Thermoactinomyces species has been covered elsewhere (Hobbs and Cross, 1983) and since the other genera contain few species, this section will deal exclusively with *Bacillus*.

4.1. Traditional Approaches

The traditional approach to the identification of *Bacillus* species relies heavily on the morphology of the spore. Species have been assigned to one of three groups depending on whether the spore is (I) oval and of the same size as the sporangium, (II) oval but of larger diameter than the sporangium thus causing distinct swelling, or (III) spherical. Familiarity with these bacteria is often necessary for successful identification using these groups but the excellent photomicrographs provided by Parry *et al.* (1983) provide a valuable reference.

Some differential features for group I strains are given in Table V. These bacteria include the *B. subtilis* group and several other common bacilli. A problematic area is the distinction of *B. amyloliquefaciens* from *B. subtilis* but this can generally be achieved on the basis of DNAase synthesis, lactose, and xylose fermentation, which is negative, positive, and negative, respectively, for *B. amyloliquefaciens* with the opposite reactions for *B. subtilis* (Priest *et al.*, 1987). However, the most reliable way to distinguish these taxa is DNA reassociation (Priest, 1981). *Bacillus megaterium* is heterogeneous (Hunger and Claus, 1981) but *B. megaterium sensu stricto* is usually recognizable due to its large size. Finally, *B. cereus*, *B. anthracis*, *B. thuringiensis*, and *B. mycoides* are difficult to separate and indeed show high DNA homology. *Bacillus thuringiensis* can be distinguished by virtue of the parasporal body and *B. mycoides* from its rhizoid growth. *Bacillus anthracis* has features that distinguish it from *B. cereus* as described in detail by Parry *et al.* (1983).

Group II is a heterogeneous collection of species (Table VI). *Bacillus macerans* and *B. polymyxa* are characterized by gas formation during sugar fermentations and *B. alvei* strains typically form motile colonies. *Bacillus circulans* presents a problem since at least 10 DNA homology groups have been recognized within this taxon (Nakamura and Swezey, 1983). Some attempt to rationalize the species has resulted in the reintroduction of *B. pabuli*, *B. lautus*, *B. amylolyticus*, and *B. validus* (Nakamura, 1984). The insect pathogens *B. larvae* (American foulbrood of honeybees), *B. lentimorbus*, and *B. popilliae* (milky disease of scarabaeid beetle larvae), which do not

Table V. Differential Characteristics of *Bacillus* Species with Ellipsoidal Spores That Do Not Distend the Sporangium (Group I)^a

	<i>B. amyloliquefaciens</i>	<i>B. anthracis</i>	<i>B. badius</i>	<i>B. cereus</i>	<i>B. fastidiosus</i>	<i>B. firmus</i>	<i>B. lentus</i>	<i>B. licheniformis</i>	<i>B. megaterium</i>	<i>B. mycoides</i>	<i>B. pumilus</i>	<i>B. subtilis</i>	<i>B. thuringiensis</i>
Cell diameter >1.0 µm	-	+	-	+	+	-	-	-	+	+	-	-	+
Parasporal crystals	-	-	-	-	-	-	-	-	-	-	-	-	d
Anaerobic growth	-	+	-	+	-	-	-	+	-	+	-	-	+
Voges Proskauer test	+	+	-	+	NG	-	-	+	-	+	-	+	d
Egg yolk lecithinase	-	+	-	+	-	-	-	-	-	+	-	-	+
Growth in lysozyme	-	+	-	+	ND	-	-	d	-	+	d	d	+
Acid from													
D-glucose	+	+	-	+	NG	+	+	+	+	+	+	+	+
L-arabinose	d	-	-	-	NG	-	+	+	d	-	+	+	-
D-xylose	d	-	-	-	NG	-	+	+	d	-	+	+	-
D-mannitol	+	-	-	-	NG	+	+	+	d	-	+	+	-
Hydrolysis of													
Starch	+	+	-	+	-	+	+	+	+	+	-	+	+
Casein	+	+	+	+	-	+	d	+	+	+	+	+	+
Nitrate reduction	+	+	-	+	-	d	d	+	d	+	-	+	+
Degradation of tyrosine	-	d	+	+	-	d	-	-	d	ND	-	-	ND
Allantoin or urate required	-	-	-	-	+	-	-	-	-	-	-	-	-
Growth in 7% NaCl	+	+	ND	d	-	+	d	+	d	d	+	+	+
Growth at													
10°C	ND	-	-	d	+	d	ND	-	+	d	+	d	d
50°C	d	-	+	-	-	-	-	+	-	-	d	d	-
55°C	ND	-	-	-	-	-	-	+	-	-	-	-	-
Utilization of													
Citrate	d	d	-	+	-	-	-	+	+	d	+	+	+
Propionate	ND	ND	-	ND	-	-	-	+	ND	ND	-	-	ND

^a+, -, d, see legend to Table IV. ND, not done; NG, no growth.

survive serial transfer in nutrient broth and are catalase-negative, are included in this group. Finally, *B. stearothermophilus* is a heterogeneous taxon within this group and probably represents at least three species-ranked taxa (Sharp *et al.*, 1980). Details for the identification of the thermophilic bacteria have been given elsewhere (Wolf and Sharp, 1981).

The group III strains contain spherical spores (Table VII). *Bacillus sphaericus* is perhaps the most common of these bacteria but, again, some seven DNA homology groups are contained within this name (Krych *et al.*, 1980). Phenotypic differentiation of these taxa is impractical at present. Other organisms with spherical spores

Table VI. Differential Characteristics of *Bacillus* Species with Ellipsoidal Spores That Distend the Sporangium (Group II)^a

	<i>B. alcalophilus</i>	<i>B. alvei</i>	<i>B. amylolycicus</i>	<i>B. azoloformans</i>	<i>B. brevis</i>	<i>B. circulans</i>	<i>B. coagulans</i>	<i>B. larvae</i>	<i>B. laterosporus</i>	<i>B. lautus</i>	<i>B. lentimorbus</i>	<i>B. macerans</i>	<i>B. macquarriensis</i>	<i>B. pabuli</i>	<i>B. pantothenicus</i>	<i>B. polymyxa</i>	<i>B. popilliae</i>	<i>B. stearothermophilus</i>	<i>B. subtilis</i>
Catalase	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Anaerobic growth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges Proskauer test	ND	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in lyszyme	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from:																			
D-glucose	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-arabinose	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-xylose	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-mannitol	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gas from glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hydrolysis of																			
Starch	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Casein	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Formation of																			
Indole	-	+	ND	-	-	-	-	-	d	-	-	-	-	-	-	-	-	-	-
Dihydroxyacetone	-	+	ND	-	-	-	d	-	-	ND	-	-	-	ND	-	+	-	-	ND
Degradation of tyrosine	-	d	ND	-	+	-	-	-	+	ND	-	+	-	ND	-	+	-	-	ND
Nitrate reduction	-	-	+	+	d	-	d	d	+	-	-	-	-	-	d	-	-	-	-
Growth in 7% NaCl	-	-	-	-	-	d	-	-	-	-	-	-	-	-	+	-	-	-	-
Growth at																			
10°C	ND	-	ND	ND	-	d	-	-	-	ND	-	d	+	ND	-	+	-	-	ND
55°C	-	-	-	-	d	-	+	-	-	-	-	-	-	-	-	-	-	-	-
65°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Utilization of citrate	-	-	-	+	d	-	d	-	-	-	-	d	-	-	-	-	-	d	+

^a +, -, d, see legend to Table IV. ND, not done; NG, no growth.

Table VII. Differential Characteristics of *Bacillus* Species with Spherical Spores (Group III)^a

	<i>B. globisporus</i>	<i>B. insolitus</i>	<i>B. marinus</i>	<i>B. pasteurii</i>	<i>B. psychrophilus</i>	<i>B. schlegelii</i>	<i>B. sphaericus</i>
Anaerobic growth	-	-	-	+	-	-	-
Voges Proskauer test	-	-	-	-	-	-	-
Growth with lysozyme	-	-	ND	ND	-	+	d
Acid from							
D-glucose	+	-	+	-	+	-	-
L-arabinose	-	-	-	-	-	-	-
D-xylose	-	-	d	-	d	-	-
D-mannitol	-	-	-	-	d	-	-
Hydrolysis of							
Starch	d	-	-	-	+	-	-
Casein	d	-	d	-	d	-	d
Deamination of phenylalanine	+	d	ND	ND	-	ND	+
NaCl or KCl required	-	-	+	-	-	-	-
Nitrate reduction	d	-	d	+	+	+	-
Growth in 7% NaCl	-	-	d	+	d	-	d
Growth at							
5°C	+	+	+	ND	+	-	-
10°C	+	+	+	ND	+	-	-
30°C	d	-	d	+	d	-	+
40°C	-	-	-	d	-	-	d
50°	-	-	-	-	-	+	-
Utilization of citrate	-	-	-	-	-	-	d
Autotrophic with H ₂ + CO ₂ or CO	-	-	-	-	-	+	-

^a+, -, d, see legend to Table IV. ND, not done; NG, no growth.

include the psychrophiles *B. globisporus*, *B. insolitus*, *B. marinus*, and *B. psychrophilus* and the thermophilic autotroph *B. schlegelii*.

It will be apparent that, as more *Bacillus* species are introduced, traditional approaches to the identification of these bacteria become impractical. Very often it is difficult to assign an unknown to a morphological group unambiguously and within groups I and II the increasing numbers of species make routine identification problematic.

4.2. Identification Kits

Since the traditional phenotypic tests for *Bacillus* may often prove unreliable and time consuming, Logan and Berkeley (1984) adopted the API 20E and 50CH trays for the identification of aerobic spore formers. These highly standardized

materials gave rise to greatly improved test reproducibility (Logan *et al.*, 1978). API produces a limited profile index for use with this system and a more extensive frequency matrix (38 taxa) has been published (Logan and Berkeley, 1984) as have details of its construction and use (Berkeley *et al.*, 1984). The resolving power of this system is such that emetic food-poisoning strains of *B. cereus* have been differentiated from other strains after suitable statistical treatment (Logan *et al.*, 1979) but *B. amyloliquefaciens* cannot be reliably distinguished from *B. subtilis* using the standard profiles. The most serious drawback is the cost of the API 50CH trays. No other identification kits for *Bacillus* have been prepared.

4.3. Computer-Assisted Identification

Growing interest in bacilli and the increasing number of species within the genus have prompted the development of computerized identification systems. The combined API 50CH and 20E database developed by Logan and Berkeley (1981) is available as a microcomputer-based system for the identification of bacilli. The matrix comprises 78 test frequencies for 38 taxa, although identification can be attempted on the basis of a limited set of test results. The criterion used for the identification of an isolate is a Wilcox probability >0.95 (Willcox *et al.*, 1973). Although the authors claim that the system has been used successfully for the routine identification of bacilli, there are no published results giving the success rates for identification of reference or unknown strains (Bryant *et al.*, 1985).

An alternative computer-assisted identification matrix is available from this laboratory and is reproduced in slightly reduced form in Table VIII. The 30 tests were chosen from a comprehensive numerical taxonomic study of the genus (Priest *et al.*, 1988) using the DIACHAR (Sneath, 1980a) and CHARSEP (Sneath, 1979b) programs, which calculate the most diagnostic features of a taxon and separation indices for the characters in a matrix, respectively. The complete 44-taxon matrix was evaluated using the MOSTTYP program (Sneath, 1980b), which calculates the hypothetical median organism (HMO) for each taxon and then attempts to identify it. All HMOs were identified correctly, 42 with a Willcox probability >0.998 and two with a Willcox probability >0.997 .

The frequency matrix (Table VIII) is used in this laboratory for the identification of natural isolates of *Bacillus* using the MATIDEN program (Sneath, 1979a). This versatile program calculates five identification indices for an unknown isolate of which we routinely use (1) Willcox probability, (2) taxonomic distance, and (3) standard error of taxonomic distance. We consider an acceptable identification comprising a Willcox probability >0.95 with a standard error of taxonomic distance <7.0 . This latter figure is higher than is usually accepted but this is probably because the number of taxa in the matrix is considerably greater than the number of tests. A typical MATIDEN output is shown in Table IX. Details of the 30 tests used are given in Appendix 2.

In an evaluation of the matrix, 23 reference strains representing most of the currently recognized *Bacillus* species were recently obtained from the German Culture Collection (DSM). These reference strains were all correctly identified using the matrix with the exception of *B. megaterium*, *B. laterosporus*, and *B. lentus*. The reasons for these discrepancies are currently being investigated and the matrix revised where appropriate but in the meantime care should be exercised in the

Table VIII. A Frequency Matrix for the Identification of Bacilli

	<i>B. alvei</i>	Taxon 4a	<i>B. mucronatus</i>	<i>B. circulans</i>	Taxon 7	<i>B. polymyxa</i>	<i>B. amyloblasticus</i>	<i>B. cereus</i> ^b	<i>B. laterosporus</i>	<i>B. amyloblasticus</i>	<i>B. subtilis</i>	<i>B. pumilus</i>	<i>B. licheniformis</i>	<i>B. megaterium</i>	<i>B. firmus</i>	Taxon 26	Taxon 27	Taxon 28	Taxon 29	<i>B. pantothenicus</i>	<i>B. brevis</i>	<i>B. azotiformans</i>	<i>B. badus</i> ^c	<i>B. psychrophilus</i>	<i>B. sphaericus</i>	<i>B. lentus</i>	<i>B. putrefaciens</i>	<i>B. coagulans</i>	<i>B. stercorithermophilus</i>	
Length > 3 µm	95	67	70	40	99	82	99	98	67	1	1	1	1	54	1	99	1	99	25	1	27	99	99	99	1	89	1	1	99	1
Gram-positive	39	1	1	20	1	1	1	99	99	99	99	90	90	99	25	99	99	99	50	78	1	99	99	99	1	99	33	67	67	50
Spores oval	99	99	99	99	99	99	99	99	99	99	99	95	99	99	99	99	99	99	99	99	91	99	99	1	1	99	99	99	99	99
Spores central	23	1	1	20	50	64	1	99	99	99	99	95	99	99	88	99	83	99	99	1	82	1	99	99	11	99	99	67	1	1
Spores bulging	92	99	99	99	99	91	99	1	89	1	1	1	1	1	1	1	17	1	1	1	99	99	1	99	89	1	99	33	99	1
Degradation of																														
Aesculin	99	99	99	99	99	99	99	99	60	99	99	99	99	99	1	1	99	99	99	99	27	1	1	20	1	99	1	67	99	99
Casein	99	83	1	1	99	99	33	99	60	99	99	99	99	99	99	99	99	99	99	99	99	1	50	99	89	1	99	99	75	1
Hippurate	99	99	50	1	1	33	2	67	11	27	99	1	9	88	50	1	1	50	1	91	1	50	99	11	99	33	99	99	99	99
Starch	99	99	99	99	99	99	99	86	1	99	99	1	99	99	99	99	63	50	99	11	1	1	1	1	1	99	1	99	99	99
Urea	31	1	1	1	1	1	1	21	1	1	1	1	32	82	1	1	1	1	1	1	1	1	50	99	1	99	1	1	1	1
Resistance to																														
Chloramphenicol	1	99	10	1	99	9	33	15	22	11	1	99	90	9	1	99	1	1	1	67	18	1	1	1	33	1	1	1	1	1
Nalidixic acid	1	1	1	99	1	1	99	2	1	1	1	1	42	1	12	1	1	1	1	99	73	99	1	1	99	99	1	99	99	99
Polymyxin	99	17	99	80	99	99	1	99	99	99	99	99	99	99	1	1	1	1	1	1	99	1	1	1	44	1	99	1	1	1
Streptomycin	92	99	99	1	99	64	1	60	20	11	73	1	95	1	1	1	1	1	1	1	99	1	1	1	99	99	67	1	1	1
Acid from																														
Cellobiose	92	99	99	99	99	99	99	84	20	99	99	99	99	99	1	1	67	1	1	1	1	1	20	1	50	1	1	1	1	1
Fructose	1	99	99	99	99	99	99	86	60	99	99	99	99	99	12	50	67	1	1	67	73	1	1	60	1	1	99	99	99	99
Galactose	54	99	90	99	99	50	91	99	3	1	33	90	95	99	1	1	1	1	1	1	1	1	1	1	50	1	1	1	1	1
Lactose	1	99	99	99	99	91	99	2	1	99	13	45	68	27	1	1	1	1	1	1	1	1	1	1	1	1	99	1	1	1
Mannose	15	99	90	99	99	82	33	21	01	89	99	99	99	45	1	1	17	1	1	1	1	1	1	1	25	1	99	75	1	75
Raffinose	99	99	90	99	99	99	99	3	1	99	99	75	95	1	1	1	1	1	1	1	9	1	1	1	1	1	1	1	1	1
Salicin	1	99	99	99	99	99	99	91	40	99	99	99	99	99	12	1	1	1	1	11	1	1	1	1	50	1	1	25	1	25
Xylose	1	99	99	99	99	91	99	1	1	11	80	95	95	99	1	1	1	1	1	1	1	1	1	1	35	1	1	1	1	1
Utilization of																														
Citrate	1	50	1	1	1	18	1	80	11	22	87	90	95	99	1	99	50	1	99	11	45	33	1	1	33	12	99	99	99	50
Succinate	1	33	80	1	1	46	99	14	99	78	87	99	99	99	99	99	50	1	99	1	45	17	99	1	99	12	99	1	50	1
Growth at 50°C	1	17	99	60	50	1	33	1	1	89	87	60	95	1	1	33	1	1	99	1	99	99	1	50	1	1	1	99	99	99
Growth in 10% NaCl	1	1	1	1	1	9	1	40	1	89	80	99	5	1	99	50	83	99	99	99	1	1	1	1	1	12	1	33	1	1
Anaerobic growth	99	99	99	99	99	99	99	99	99	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	99	99	99	50
Nitrate reduction	1	33	99	20	1	99	99	97	99	78	99	1	99	1	99	99	1	1	1	89	91	99	50	80	44	37	67	99	99	99
Oxidase reaction	99	99	99	1	99	1	99	69	11	1	1	10	10	1	1	1	17	1	1	22	1	83	25	20	89	99	1	1	1	1
Voges-Proskauer test	1	1	1	1	1	99	1	91	1	99	99	99	19	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

^aUnnamed taxa comprise the following strains: taxa 4 and 7, *B. circulans*; taxa 26–29, isolates from marine muds.

^b*B. cereus* includes *B. mycoides* and *B. thuringensis*.

^c*B. badus* includes "*B. freudenreichii*."

Table IX. Example of the Output Provided by the MATIDEN Program for the Identification of an Unknown Strain against the Frequency Matrix

INPUT THE CHARACTER VALUES OF THE UNKNOWN			
REFERENCE NUMBER OF UNKNOWN IS S222 BEST			
IDENTIFICATION IS <i>B. MEGATERIUM</i>			
SCORES FOR COEFFICIENT: 1 (Willcox probability), 2 (Taxonomic distance), 3 (Standard error of taxonomic distance)			
	1	2	3
<i>B. MEGATERIUM</i>	.999	.324	4.665
<i>B. LENTUS</i>	$.673 \times 10^{-6}$	.481	6.554
<i>B. CIRCULANS</i>	$.142 \times 10^{-7}$	.514	9.256
CHARACTERS AGAINST <i>B. MEGATERIUM</i>			
Character	% in taxon	Value in unknown	
NALIDIXIC ACID	1	+	
GROWTH 10% NaCL	99	-	
ADDITIONAL CHARACTERS THAT ASSIST IN SEPARATING <i>B. MEGATERIUM</i> FROM:			
<i>B. LENTUS</i> :	NONE		
<i>B. CIRCULANS</i> :	NONE		

interpretation of identifications to these taxa. To extend the evaluation, 58 *Bacillus* strains were isolated from soil and estuarine mud samples. Of these, 34 (58%) were identified to 12 different taxa. If the Willcox probability score alone was used (i.e., >0.95), 41 (70%) could be identified. Some representative results are shown in Table X. Although this matrix has yet to be optimized, in its present form it seems to be useful for the identification of environmental isolates of *Bacillus*.

4.4. Chemotaxonomic Analyses for Identification

Of the various chemotaxonomic data available (see Chapter 2, Section 4.3), fatty acid composition seems to hold the most potential for identification purposes. Using standardized growth and analytical conditions, reproducible fatty acid profiles suitable for multivariate statistical analysis can be obtained (O'Donnell, 1985), and thus it should be possible to develop identification systems. These sophisticated methods have proved useful for the classification and identification of streptomycetes (Saddler *et al.*, 1987) and a microbial identification system using gas chromatography (GC) analysis of methyl esters of cellular fatty acids is marketed by Hewlett-Packard (HP5898A system). The standard library provided with the machine contains profiles for 17 common bacilli but individual reference libraries could be constructed. The production of the profile takes about 60–90 min (60-min

Table X. Examples of Identification Scores for Unknown Strains Isolated from Garden Soil and Estuarine Muds

Strain ^a number	Identification	Identification scores	
		Willcox probability	Standard error of taxonomic distance
EL1	<i>B. pumilus</i>	0.999	4.20
EL7	<i>B. megaterium</i>	0.999	6.04
EL8	<i>B. subtilis</i>	0.998	1.09
EL10	Taxon 27	0.984	3.02
EL13	<i>B. cereus</i>	0.999	5.35
EL18	<i>B. polymyxa</i>	0.986	5.23
T5	<i>B. licheniformis</i>	0.999	5.67
T10	<i>B.adius</i>	0.999	3.26
T20	Taxon 29	0.966	5.49
EL20	Unidentified	0.997	8.34
T25	Unidentified	0.389	17.15
T26	Unidentified	0.543	7.63

^aEL strains isolated from garden soil; T strains from estuarine muds.

sample preparation time), although when samples are prepared and analyzed in batches the actual preparation time per sample is quoted at less than 4 min.

4.5. Identification Using Pyrolysis Techniques

Pyrolysis, controlled thermal degradation in an inert atmosphere, can be used to produce a complex mixture of low molecular weight volatile compounds from a sample. The mixture can be separated using GC to produce a fingerprint known as a pyrogram. This technique of pyrolysis gas chromatography (Py-GC) has been used successfully for the identification of various groups of microorganisms including bacilli (reviewed by Gutteridge and Norris, 1979; O'Donnell and Norris, 1981). The complexity of the pyrogram requires sophisticated statistical procedures for data processing but the application of multivariate analysis has been successful (O'Donnell and Norris, 1981; Gutteridge *et al.*, 1985). For example, canonical variates analysis of Py-GC data separated *B. subtilis* from *B. amyloliquefaciens* (O'Donnell *et al.*, 1980a) and distinguished *B. thuringiensis*, *B. mycoides*, and *B. cereus* (O'Donnell *et al.*, 1980b).

However, there are problems associated with this technique, particularly concerning reproducibility and standardization. Degeneration of the chromatographic column ultimately occurs and column replacement often gives results that are markedly different from its predecessor due to difficulties in standardizing column performance. As a result of these problems, attention has focused on pyrolysis mass spectrometry (Py-MS) as an alternative procedure.

Pyrolysis-MS separates the pyrolysis fragments on the basis of their mass/charge ratio (m/z) using a mass spectrometer. The technique is faster than Py-GC, has fewer problems with reproducibility, but is much more expensive. Never-

theless, low-cost commercial systems with automated sample input are being developed. The pyrograms or mass spectra are generally analyzed using multivariate statistics (reviewed by Gutteridge *et al.*, 1985).

To date, Py-MS has not been used extensively for identification of bacilli. Shute *et al.* (1984) examined 53 strains of the "*B. subtilis* group" using a Pyromass 8-80 machine, a pyrolysis mass spectrometer specifically designed for fingerprinting complex samples. Strains of all four species (*B. amyloliquefaciens*, *B. pumilus*, *B. licheniformis*, and *B. subtilis*) could be distinguished and when a small database of these strains was constructed and challenged with seven "unknowns" all were correctly identified (Berkeley *et al.*, 1984). Since the more extensive studies with Py-GC have indicated the value of pyrolysis for identification purposes, it seems likely that the greater reproducibility of Py-MS will have great potential for automated identification of bacilli.

5. CONCLUSIONS

There seems little doubt that the demands of modern biotechnology for novel products will encourage more numerous and extensive screening programs and consequently more new aerobic, endospore-forming bacteria will be described. Insect pathogenic bacilli with improved virulence, novel enzymes from thermophiles and alkaliphiles, antibiotics and other small molecules with pharmacological activities are just some of the potential products from as yet undiscovered spore-forming bacteria. These new bacteria will have interesting biological properties and methods for their classification and identification will be required. Such methods should be inexpensive, rapid, and reliable and this brief review has described some of the approaches currently available. Future developments will probably occur in two main areas: the reduction in cost of automated systems such as Py-MS thus making them realistic alternatives for the medium-sized laboratory, and the development of reliable and rapid tests, such as enzyme-based tests, that can be incorporated into frequency matrices and used for probabilistic identification using a microcomputer. Certainly the next decade will witness dramatic advances in the speed and reliability with which we identify these important bacteria.

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EUGENIO FERRARI and JAMES A. HOCH

1. INTRODUCTION

Bacillus subtilis has been a paradigm of gram-positive genetics for almost 30 years. It has virtually all of the classical genetic tools of *Escherichia coli* with the exception of conjugation and has an exceptionally well-developed transformation system with unique features. Furthermore, vectors for molecular genetic analyses of *B. subtilis* are capable of the majority of molecular tricks exploited in its more intensely studied gram-negative relative. In this chapter we will attempt to put in perspective those techniques of classical and modern genetics that have proven most useful for genetic analyses of *B. subtilis* and for studies of the control of gene expression in this organism. This viewpoint will encompass those methodologies proven most useful in our practical experience and is not intended to be exhaustive.

2. MECHANISMS OF GENETIC EXCHANGE

2.1. Transformation

Historically, transformation was the first genetic exchange tool studied. From 1928, when Griffith was able to transfer the virulence character in *Pneumococcus*, almost 20 years passed before it was shown that DNA was responsible for the transferred genetic modification (Avery *et al.*, 1944). *Bacillus subtilis* transformation was discovered by Spizizen (1958) and since then has been the most widely used system for genetic manipulation in this organism. Transformation, as defined here, refers to the ability of the bacterial cell to take up DNA molecules from solution. The donor DNA can be either of chromosomal or plasmid origin, and, after entering the recipient cell, has to be able to modify at least part of the cell genotypic and phenotypic characteristics which are transferred to its progeny. There are basically two ways by which transformation can be accomplished in *Bacillus*. The classical

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method requires that the recipient cell be in a particular metabolic state called competence. Transformation through competence is widely used to perform chromosomal transformation as well as plasmid transformation, but it is available only for certain members of the *Bacillus* genus, e.g., *B. subtilis* and *B. licheniformis*. A more recent method introduces DNA into protoplasts through partial permeabilization of the membrane with high molecular mass polyethylene glycol. Although this transformation system should allow transformation of virtually any *Bacillus* species, it may be of limited application because only plasmid DNA seems to be taken up efficiently. There is only one report of successful protoplast transformation using chromosomal DNA as a donor (Levi-Meyrueis *et al.*, 1980).

2.1.1. Competent Cell Transformation

A great deal of work has been done trying to elucidate the transformation process in bacteria and it would go beyond the purpose of this review to attempt a detailed explanation of the proposed mechanism. A more interested reader is referred to a number of excellent and extensive reviews which delve into the physiology and biochemistry of this process (Dubnau, 1976, 1982; Venema 1979; Smith and Danner, 1981; Dressler and Potter, 1982).

Competence is a metabolic state in which a cell acquires the ability to take up naked DNA from the environment, transport it as a single-stranded molecule through the cell wall-membrane complex, and incorporate it into the chromosome. Exactly what happens to the cell when it develops competence and how this state is induced are still unclear. Several growth media have been developed to induce competency (Anagnostopoulos and Spizizen, 1961; Wilson and Bott, 1968; Stewart, 1969; Dubnau and Davidoff-Abelson, 1971). All stem from the original Spizizen procedure (1958) but seem to work with differing efficiencies in different strains and in different laboratories. Probably this reflects nonphenotypical differences in the genotype of the strains used and/or trace amounts of elements present in the water or the reagents used in the preparation of the medium. The maximum level of competent cells reached in a population is about 10–20% (Somma and Polsinelli, 1970). Competent cells can be separated from the noncompetent ones by fractionation in urographin or renographin gradients (Cahn and Fox, 1968; Hadden and Nester, 1968).

Competence is thought to be associated with sporulation (see Chapter 8), given the observation that some Spo^- mutants, in particular those bearing *spoOA* alleles, have a lower transformation efficiency than the wild type (Spizizen, 1965). Whether this correlation is direct or due to a secondary effect of the *spoOA* genotype is still unclear and debatable. A competence factor has been shown to be produced and released into the medium by *Streptococcus* and *Hemophilus* competent cells, and supernatant from *Streptococcus* competent cells can readily induce competence when mixed with noncompetent cells (Smith and Danner, 1981). Competence factor has been suggested also in *Bacillus*, but its presence has never been proven (Akrigg and Ayad, 1970).

Competent *Bacillus* cells can bind very efficiently several molecules of double-stranded DNA (Dubnau and Cirigliano, 1972b) and, contrary to what happens in *Hemophilus* (Sisco and Smith, 1979), no sequence specific recognition binding site seems to be required. Heterologous DNA, such as T7 DNA, can in fact be bound

and transported inside the cell as efficiently as homologous DNA (Dubnau, 1976, 1982). The double-stranded DNA bound by the cell is cleaved by nucleases into fragments with an average size of 20–30 kb (Dubnau and Cirigliano, 1972a; Arwert and Venema, 1973) and transported inside the cell as single-stranded DNA (Piechowska and Fox, 1971; Dubnau and Cirigliano, 1972a). Mutants impaired in the binding of the DNA have been described by Mulder and Venema (1982). From these studies it has been possible to identify a 75,000-Da protein complex involved in the binding and uptake of the donor DNA (Smith *et al.*, 1985). Once inside the cell the single-stranded DNA pairs with the chromosome at a region of homology and replaces the identical strand. The efficiency of the replacement is directly proportional to the homology between the donor and the recipient DNA as shown by transforming *B. subtilis* with DNA from other *Bacillus* species (Marmur *et al.*, 1963; te Riele and Venema, 1982).

The average length of the fragments of the donor DNA is also an important factor for obtaining high efficiencies of transformation. DNA with a molecular mass of less than 5×10^6 Da is bound poorly and therefore gives low transformation efficiencies (Bodmer, 1966). The mechanisms by which the integration steps are accomplished are not fully elucidated. DNA replication does not seem to be active since the metabolic state of the competent cell is low (McCarthy and Nester, 1967) nor does it seem to be necessary as shown by studying transformation in the presence of HPUra, which blocks the replication of the DNA by acting on DNA polymerase III (Dubnau and Cirigliano, 1973; Levin and Landman, 1973). An interesting hypothesis proposed by Dubnau (1976) suggests that short regions of DNA with an open configuration may be the spots where the single-stranded incoming DNA binds and progressively pairs with the complementary strand as the open configuration moves along the chromosome. Both *in vivo* (Bodmer and Ganesan, 1964; Dubnau and Davidoff-Abelson, 1971) and *in vitro* analysis of heteroduplexes (Strauss, 1970) support this interpretation. If replication of the DNA is not involved, DNA repair functions are certainly needed for the processing of DNA during the pairing and incorporation in the chromosome. Several recombination deficiency mutations (for review, see Mazza and Galizzi, 1978) isolated as conferring sensitivity to mitomycin C or methylmethanesulfonate and involving at least 10 different genes have been shown to affect both the repair and recombination processes (Mazza *et al.*, 1975; Dubnau, 1976; Yasbin, 1977a,b).

Transformation of competent cells with plasmid DNA is not as efficient as transformation of the same cells with chromosomal DNA. The ratio between transformants obtained and number of molecules of plasmid DNA is 10^{-3} – 10^{-4} (Gryzcan *et al.*, 1979). This ratio probably reflects the composition in monomer versus multimer of the plasmid population used in a particular experiment. In fact, as shown by Canosi *et al.* (1978) and Mottes *et al.* (1979), monomeric plasmid has no or little transforming activity, while multimers can readily transform *B. subtilis* competent cells at very high efficiency. A model for plasmid transformation has been suggested by several investigators (Canosi *et al.*, 1981; de Vos *et al.*, 1981) and extensively described by Dubnau (1982). The plasmid DNA is thought to bind to the competent cells, to be cleaved and both strands transported inside the cell as single-strand units, in a manner similar to that of chromosomal transformation. Once inside the cell the opposite strands pair, recircularize due to the presence of redundant termini, and reconstitute an intact plasmid. This model would also

explain the higher efficiency obtained by monomers carrying a fragment of DNA homologous to the chromosome (Canosi *et al.*, 1981) or to plasmid already resident in the recipient cell (Contente and Dubnau, 1979). In the latter case the donor plasmid would be rescued due to the homology with DNA of plasmidic or chromosomal origin already resident inside the recipient cell.

2.1.2. Protoplast Transformation

A more efficient way of introducing plasmids in *Bacillus* is through the protoplast transformation method described by Chang and Cohen (1979). With this procedure most of the problems created by the competent cells are avoided. The monomeric form of the plasmid gives the same transformation efficiency as multimers, and also ligation mixtures can be used (see Chapter 6). Due to the high efficiency reached by the system, up to 10^7 transformants/ μg of DNA (Chang and Cohen, 1979), shotgun cloning is also possible. In certain cases it is possible to devise special media to select or at least screen directly on the regeneration plates for the desired clone (Corfield *et al.*, 1984). Although important for cloning in *B. subtilis*, this procedure is even more important for the cloning or transfer of plasmids in certain industrially exploited strains, such as *B. amyloliquefaciens* and *B. licheniformis* (Trosch and Wollweber, 1982). Protoplast regeneration and transformation frequencies in these two species don't seem to be as efficient as in *B. subtilis*. Lysozyme concentration and length of treatment are more critical than with *B. subtilis* but the preliminary results obtained seem to hold good promise. Transfer of plasmid through protoplast fusion seems also to be very promising (Aksamatsu and Sekiguchi, 1983) and it seems to be more efficient than plasmid transformation, at least with some industrial *Bacillus* species (E. Ferrari, unpublished results).

Protoplast transformation seems to be particularly useful for bacterial species which do not have a natural competence system such as *B. megaterium*, (Brown and Carlton, 1980), *B. thuringiensis* (Martin *et al.*, 1981; Fischer *et al.*, 1984), and *B. stearothermophilus* (Imanaka *et al.*, 1982).

2.2. Transduction

Phage-mediated transduction has played a major role in the linkage analysis of *B. subtilis* allowing the elucidation of the genetic structure of its chromosome. The primary bacteriophage used in these studies has been PBS1 and its relatives (Takahashi, 1961). This phage is capable of packaging at least 250 kilobase pairs of DNA representing 6–8% of the chromosome. The large fraction of the chromosome transferred by this phage resulted in PBS1 transduction being the primary method of analyzing widely separated genetic markers which ultimately led to a circular genetic map (Lepesant-Kejzlarova *et al.*, 1975). The combination of PBS1 transduction and DNA-mediated transformation, the latter of which is best used for fine-structure genetic analysis of closely linked genes, has made *B. subtilis* the gram-positive equivalent of *E. coli*.

Many different bacteriophages have been described for *B. subtilis* (Hemphill and Whiteley, 1975; Rutberg, 1982). Some of these, such as SP-10 (Thorne, 1962) or SPP1 (Yasbin and Young, 1974; Ferrari *et al.*, 1978), have transducing properties,

although they have fallen into disuse for *B. subtilis*. SPP1 might be useful for plasmid transduction (Canosi *et al.*, 1982; Deichelbohrer *et al.*, 1985). A number of phages which can infect and transduce fragments are available for almost all the members of the *Bacillus* genus. For certain *Bacillus* species, such as, for example, *B. thuringiensis*, transduction together with protoplast fusion (see below) is so far the only system available for genetic analysis (Carlton and Gonzalez, 1985). This is due to the low efficiency of protoplast transformation and to the lack of natural competence. Thorne and co-workers (Barsomian *et al.*, 1984; Ruhfel *et al.*, 1984) were able to transfer plasmids among several *Bacillus* species with the aid of phage CP-51. PBS1-transducing lysates are relatively easy to prepare (Hoch *et al.*, 1967) and remain stable for years. The only limitation to PBS1 is that it requires motile cells to attach and infect, and thus cannot be used with nonmotile mutants. Since phages related to PBS1 are distributed on a worldwide basis (Birdsell and Hoch, unpublished results), it seems likely that a PBS1-like phage could be found for virtually all *Bacillus* species.

The mechanism by which the DNA fragments carried by bacteriophage PBS1 integrate into the chromosome has not been studied as extensively as in the transformation process. It is evident from genetic studies done with Rec^- mutants that the two pathways have certain steps in common. In fact, all the Rec^- mutations which affect transformation affect also transduction (Mazza *et al.*, 1975; Mazza and Galizzi, 1978) with the exception of *recG* and *recA* (Hoch *et al.*, 1967). The function coded by these latter genes may be used only in transformation. The fact that transduction is affected by the host restriction system (Trautner *et al.*, 1974) while competence-induced transformation is not may indicate that transducing DNA is transported across the membrane in a double-stranded form.

2.3. Protoplast Fusion

One potential way to analyze *Bacillus* genetically is via protoplast fusion (Schaeffer *et al.*, 1976). It has been used with some success in several bacterial species and it overcomes barriers such as lack of natural transformation and/or transduction systems (Hopwood, 1981). Many factors are involved in the successful production and the subsequent fusion of protoplasts. Also the analysis of the fusants may be complicated by several variables. The reader is referred to recent comprehensive reviews by Hopwood (1981) and by Hotchkiss and Gabor (1985) which analyze the methodology in depth. Only a brief description of the methodology and some of the inherent problems are referred to here.

The procedure for fusion consists of preparing protoplasts of the strains to be fused through lysozyme treatment, mixing the cells together in the presence of high molecular mass polyethylene glycol (PEG) and plating the mixture on appropriate regeneration plates. Many factors can affect the success of the fusion and most of these are also of importance for protoplast transformation. The time of exposure to lysozyme can influence tremendously the recovery of regenerated protoplasts since excessive exposure may remove all traces of peptidoglycan, which is needed as a primer for cell wall regeneration. Certain species, such as *B. subtilis*, do not seem to be affected by the amount (up to 10 mg/ml) or the length of exposure (up to 1–2 hr) to lysozyme (Chang and Cohen, 1979). Other species, such as *B. licheniformis* and *B. amyloliquefaciens*, need to be exposed to an amount of lysozyme not exceeding 1

mg/ml and for not more than 15–20 min if high frequencies of regeneration are to be achieved (Trosch and Wollweber, 1982; Akamatsu and Sekiguchi, 1983). The presence of detergents has to be painstakingly avoided. Even traces in the glassware or in any of the reagents used dramatically disrupts the entire process due to the excessive detergent sensitivity. In the case of protoplast transformation, Chang and Cohen (1979) suggest extensive dialysis and several ethanol precipitations of the donor DNA in order to eliminate any carryover of detergents used during DNA preparation. Polyethylene glycol treatment, which seems to promote fusion through the formation of bridges between protoplasts in close contact, need not exceed 1–2 min. Longer treatment will result in a poor regeneration frequency due to the toxic effect exerted by PEG on the protoplasts. The regeneration process has not been completely clarified. Generally, the presence of Mg^{2+} ions and gelatin or other protein sources, i.e., bovine serum albumin, in a solid medium which offers osmotic protection is required (DeCastro-Costa and Landman, 1977). Slightly modified media might be needed for the regeneration of bacilli other than *B. subtilis* to increase the efficiency of regeneration.

The choice of an appropriate selection medium is also important to obtain an even representation of all the classes of fusants. When counterselection is employed there are often biases (Hotchkiss and Gabor, 1985) due to not yet clarified metabolic effects. Direct selection, although useful in certain cases when a particular class of recombinants is desired, might lower the recovery efficiency of certain classes of fusants. Plating of the fusants in a nonselective medium and analyzing the regenerated protoplast population after several rounds of subcloning yields recombinants at higher frequency (Gabor and Hotchkiss, 1982). In this type of analysis, according to Karmazyn-Campbell *et al.* (1985), cell wall regeneration lowers the number of prototrophic clones obtainable after a fusion. This observation would be in agreement with the suggested role played by cell wall chromosome attachment sites (Hotchkiss and Gabor, 1985), which could also be responsible for some of the unexplained phenomena such as inactivation of one of the two chromosomes, and nonreciprocal recombination and segregation observed in protoplast fusion of the bacilli. After protoplast fusion, in fact, a high portion of the colonies regenerated in the absence of selective pressure carry both parental chromosomes (Hotchkiss and Gabor, 1980; Guillen *et al.*, 1983) and only one of them is in a transcriptionally active state.

2.4. Evaluation of Gene Transfer Mechanisms for Genetic Studies

2.4.1. Transformation

Transformation is certainly the most versatile and efficient system for genetic studies in *B. subtilis* and certain *B. licheniformis* strains. The high efficiency with which this process is carried out allows an easy and reproducible way to engineer new strains even in mutants where PBS1 transduction is not possible, or the transformation itself is greatly impaired. In fact, due to the high number of molecules of DNA, up to 50, that can be bound by a single competent cell (Dubnau and Cirigliano, 1972b; Singh, 1972) and to the fact that almost all the molecules entering the cell can be integrated into the chromosome (Bodmer, 1966; Somma and Polsinelli, 1970), two or more unlinked markers can be easily introduced into a recip-

ient cell. This phenomenon, called congression, can cause problems when transformation is used for gene-mapping purposes. False linkage of 1% or more between markers located several hundred kilobase pairs apart can be observed when high concentrations of DNA are used. So particular care has to be taken when using transformation for gene mapping to use nonsaturating concentrations of donor DNA, i.e., less than 1 $\mu\text{g/ml}$.

Due to the relatively low molecular mass of the DNA entering the cell transformation is a particularly useful system for fine-structure chromosomal mapping. Distances between closely linked genes or within mutations in the same gene can be calculated. On the other hand, this feature does not allow linkage analysis of genes distant by more than 15–20 kilobase pairs. An evaluation of the relationship between frequencies of cotransformation or cotransduction and physical distance was described by Henner and Hoch (1980). New data are available for the *AroI-AmyE* region (Ferrari, E. *et al.*, 1985), for the *trp* region (Henner *et al.*, 1984), for the *spoOB* region (Ferrari, F. A. *et al.*, 1982), and for the region around the origin of replication (Moriya *et al.*, 1985; Ogasawara *et al.*, 1985). The tryptophan region is probably the best characterized. We attempted to correlate the map distances as determined by Anagnostopoulos and Crawford (1967) and the sequence of the same region by Henner *et al.* (1984). The outcome of this analysis indicates that in transformation 1 map unit (i.e., 1% of recombination or $[1\text{-cotransfer}] \times 100$) corresponds on average to about 100 base pairs. Nevertheless, only when the exact location of the different mutations used in the mapping studies are determined within a gene will it be possible to establish precisely the relation between map units and base pairs. Particular precautions need to be used in this type of consideration because map units observed between two markers in the different crosses are not necessarily reproducible, and the linkage between two markers may vary depending on the particular marker selected and marker effects, e.g., abnormally high or low integration of specific alleles, can occur. However, map unit values serve as a guide for estimating physical distance from genetic data and may be particularly useful in developing cloning strategies.

2.4.2. Transduction

The major advantage that PBS1 transduction offers over transformation is the fact that it can mediate the transfer of large chromosomal fragments, allowing the cotransfer of markers as far away as 250 kilobases. This, as mentioned above, makes the genetic mapping of genes fairly easy in an organism which lacks proven and reliable conjugation. Minor drawbacks are the low efficiency of the system when compared to transformation and the fact that it doesn't allow the refined mapping capabilities offered by chromosomal transformation. A kit consisting of nine reference strains prepared by Dedonder and co-workers (1977) carrying convenient markers scattered on the chromosome is available for mapping purposes. The markers were chosen in such a way as to be linked in transduction and allow testing of the entire chromosome with one round of transduction.

2.4.3. Protoplast Fusion

Protoplast fusion has been successfully used as a tool for genetic mapping in *Streptomyces* (for review, see Hopwood, 1981) and in *Staphylococcus* (Stahl and Pattee,

1983a,b). In certain species of *Bacillus* which almost completely lack a transformation system, such as *B. megaterium*, attempts have been made to use the protoplast fusion as an alternative to transduction for genetic analysis (Fodor and Alföldi, 1976; Fleischer and Vary, 1985). Unfortunately, the genetic analysis of the outcome is somewhat complicated by phenomena such as stability of the diploids in the population, nonreciprocal recombination with segregation of one of the recombinant parents but not the other, and complete or partial inactivation of one of the two chromosomes present in the diploid. For the reasons mentioned above, a better understanding of the entire process will be required before it can be used with confidence as a genetic tool. In our opinion the best immediate use of protoplast fusion in *Bacillus* is for the genetic manipulation of industrially important strains which seem to be deprived of the more practical transformation and transduction systems. If any work of this type has been attempted to date, as is the case for members of the genus *Streptomyces* it is not of general public knowledge. Only one report of a successful application of this technique to engineer a novel industrial strain is available (Sanders *et al.*, 1985). The possibilities offered by this technique are of great importance both for basic research and for the industrial exploitation of members of the genus.

3. METHODS OF MUTAGENESIS

3.1. Classical Techniques

Bacillus subtilis responds to all of the mutagenesis techniques usually employed for prokaryotes. In addition, a highly efficient mutagenesis technique using outgrowing spores has been developed that is probably applicable to most sporulating organisms (Balassa, 1969). In this technique suspensions of dormant spores were heat-activated and allowed to germinate at 37°C in rich media containing chloramphenicol to prevent protein synthesis. A mutagen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was added for a period of time and the cell suspension was washed to remove mutagen and chloramphenicol. The mutagen-treated, germinated spores were then plated for mutant detection. The germinated spore suspension maintained stable titer even after storage at 4°C. The proportion of sporulation defective mutants in the plated colonies was very high, sometimes yielding more than 25% nonsporulating mutants. In some experiments, the level of auxotrophs was of the order of 5%. This effective method has been buried in the literature and has not been exploited to its full potential. Parameters such as the length of mutagen treatment, type of mutagen, type of antibiotic, have not been explored.

3.2. Integrative Vectors and Transposons

The ability to transform *B. subtilis* with naked DNA at high frequency has allowed the development of two versatile systems for mutagenesis and molecular genetic analysis: integrative vectors and transposons. Transposons have been exploited for genetic studies of *E. coli* and relatives and their properties are well known. The streptococcal transposon Tn917 (Tomich *et al.*, 1980) specifying eryth-

romycin resistance has been adopted for use in *B. subtilis* (Youngman *et al.*, 1983). The basic transposon is contained within the plasmid pTV1 (see Chapter 6, Figure 4). This plasmid specifies chloramphenicol resistance on a temperature-sensitive replicon such that replication occurs freely at 30°C but not at 47°C. If cells containing this plasmid are plated at 47°C, with selection for macrolide, lincosamide, and streptogramin type B (MLS, erythromycin and lincomycin) antibiotics resistance, the colonies that appear are due to transposition of the MLS resistance determinant onto the chromosome. The transposition of this element is not random; about 95% of the resistant colonies have the transposon inserted near the terminus of the chromosome. The other 5% are presumably inserted at random in the rest of the chromosome. *In vitro* manipulation of the transposon has led to transposons that generate transcription fusions to either β -galactosidase or chloramphenicolacetyl-transferase, or both, upon transposition (see Chapter 6, Fig. 8; Youngman *et al.*, 1985; Perkins and Youngman, 1986). These allow the direction and, in some cases, the temporal nature of transcription within the interrupted gene to be determined (see Chapter 8, Section 4). Thus transposon mutagenesis has proved to be very versatile, allowing transcriptional fusions as well as ordinary transpositions.

The exploitation of integrative vectors in prokaryotes is almost unique to *B. subtilis* and results from the extensive use of *E. coli* for cloning of *B. subtilis* genes. Integrative vectors are basically *E. coli* plasmids containing an antibiotic resistance gene capable of expression in *B. subtilis*. pJH101 (Fig. 1) is typical of this class of vectors, consisting of the *E. coli* plasmid pBR322 with a chloramphenicol resistance gene from a gram-positive replicon (Ferrari, F. A. *et al.*, 1983). Integrative vectors are incapable of replication in *B. subtilis* and cannot integrate in this organism without a region of homology to the *B. subtilis* chromosome. With a region of homology these vectors integrate into the chromosome by a Campbell-type mecha-

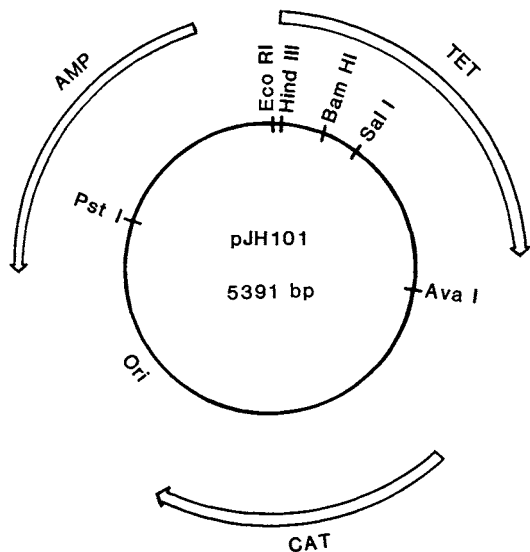


Figure 1. Restriction map of the integrative vector pJH101 (Ferrari, F.A. *et al.*, 1983). This vector confers ampicillin, tetracycline, and chloramphenicol resistance to *Escherichia coli* and chloramphenicol resistance to *Bacillus subtilis* when integrated.

nism, resulting in duplication of the region at either end of the vector. Several papers describe the use of these types of plasmids and their mode of integration (Stahl and Ferrari, 1984; Young, 1984; Albertini and Galizzi, 1985). Integrants of this type are quite stable in the absence of antibiotic selection as long as the plasmid is not deleterious to the growth of the cell.

Mutagenesis experiments using plasmids like pJH101 are carried out by cloning into them fragments of the *B. subtilis* chromosome. Single- or double-restriction enzymes sites may be used as the ampicillin and tetracycline resistance genes of pJH101 serve to monitor of the efficiency of cloning. Ligation mixtures are transformed into *E. coli* and transformants are selected using the noninactivated antibiotic resistance marker. Transformants are pooled in convenient numbers and the plasmids extracted together. Such pools can be maintained for years as frozen stocks and serve as a source for transformation into competent *B. subtilis*. Upon transformation all of the chloramphenicol-resistant transformants result from integration of the vector but the proportion of auxotrophs obtained will vary according to the size of fragments cloned and the size of the target gene(s). Since a duplication is generated upon integration, those cloned fragments that are wholly contained within a gene or a single transcription unit will generate auxotrophs. Most of the fragments that stop within a gene yet extend beyond either of its ends, are non-mutagenic since one good copy of the gene is generated in the integration event. Thus transposons should generate more mutations per integration event than integrative vectors.

Second generation integrative vectors have recently been constructed by M. Perego (unpublished). Figure 2 shows the integrative vector pJM102 which contains the same chloramphenicol resistance gene as pJH101 inserted in pUC18. A similar plasmid, pJM103, derived from pUC19 is also available. These allow insertion of a variety of fragments in the multiple cloning site with inactivation of the Lac phenotype in *E. coli*. In recent experiments using pJM102 as a mutagenic agent by cloning *Taq* I fragments in the *Acc* I site, the yield of auxotrophs was 1% and the yield of sporulation defectives was 10% among the chloramphenicol-resistant transformants of a *B. subtilis* strain. This compares favorably with the best classical mutagenesis experiments. These integrants may also serve as a means to clone the mutated genes by excision of the vector and adjacent sequences and subsequent transformation into *E. coli*.

Integrative vectors for the generation of translation fusions to β -galactosidase have been developed (Ferrari, F. A. *et al.*, 1985). pJF751 (Fig. 3) differs from pJH101 in that the tetracycline gene has been replaced by the promoterless *lac* gene originally described by Casadaban *et al.* (1980). *Lac* fusions may be generated at the *Eco* RI, *Sma* I, or *Bam* HI site. A derivative of this plasmid for transcriptional fusions is under development (M. Perego, personal communication).

3.3. Evaluation of Mutagenesis Methods

Both transposons and integrative vectors possess advantages and disadvantages, and the choice of one over the other depends on the projected use. Either system works for direct mutagenesis. Transposon Tn917 suffers from a hot spot of transposition that consumes greater than 90% of the transpositions. Integrative vectors are more random but transformation frequency falls drastically as the size

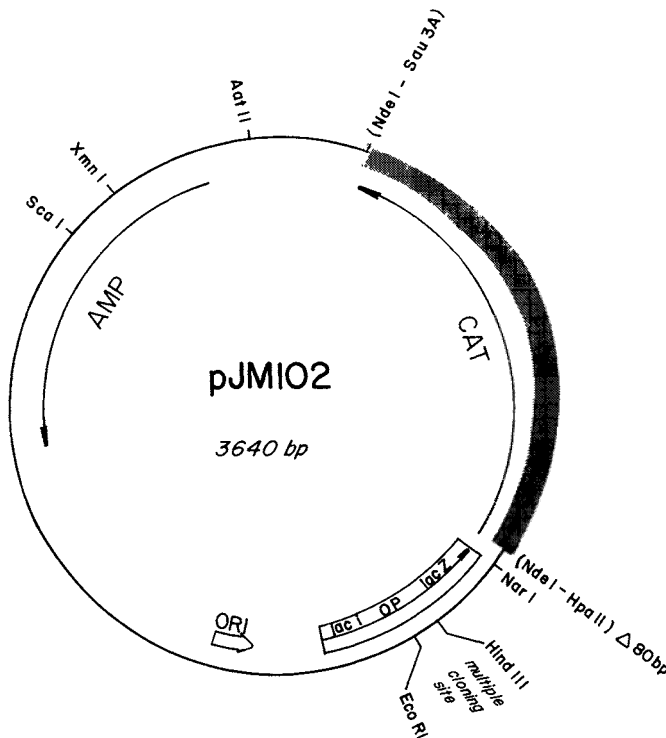


Figure 2. Restriction map of the integrative vector pJM102. This plasmid contains a chloramphenicol resistance gene in the plasmid pUC18 (Yanish-Perron *et al.*, 1985).

of the cloned fragment decreases resulting in nonrandomness from preferential integration of plasmids carrying longer fragments. However, fragments as small as 150 base pairs have been successfully integrated. Either system provides a genetic marker for mapping purposes along with transcriptional and translational fusion capability. Cloning the mutated gene is a two-step process with transposon Tn917 and a one-step process with integrative vectors. Cloning in either system requires

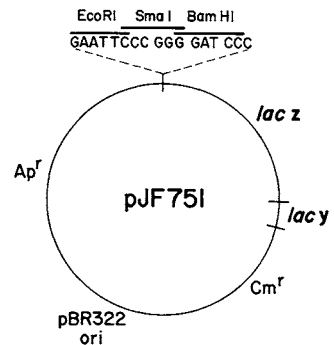


Figure 3. Restriction map of the integrative vector pJF751 for translational fusions to β -galactosidase. This vector is a derivative of pJH101 containing the β -galactosidase gene of pMC1403 (Casadaban *et al.*, 1980).

the use of *E. coli* and inherent problems using this organism, such as lethality of cloned genes and replication inhibition properties of strong promoters, cannot be easily avoided.

4. COMPLEMENTATION AND DOMINANCE

There is no formal equivalent of the F factor in *Bacillus* and therefore this major system of gram-negative complementation does not exist. Although a system of complementation using SP β specialized transducing phage has been described (Zahler and Korman, 1981), it has not gained widespread popularity. Classical complementation of enzymatic deficiencies can probably be carried out with cloned genes on virtually any replicating vector regardless of copy number. Some sporulation genes, however, are complemented only by low and not high copies of their wild-type genes (Yoshikawa *et al.*, 1985; see Chapter 8).

Dominance is a reflection of the subtle interplay of regulatory elements among themselves or with their targets. For that reason it is important to balance the concentrations of these elements to correctly interpret the results obtained. Integrative vectors are useful for such studies because they generate a duplication of the fragment of interest and, as long as the fragment contains an intact copy of the gene analyzed, plasmid and recipient alleles of a gene are present in equimolar concentrations. Special integrative vectors carrying a fragment of bacteriophage SP β that allow integration at a distance from the genes being analyzed may be useful in certain instances (Ferrari and Hoch, 1983). New vectors for complementation and dominance analysis could be constructed using the same strategy described by Shimotsu and Henner (1986) to analyze the regulation of the tryptophan operon. In order to avoid plasmid amplification, which often occurs when the integration event is mediated by a single crossing over, these authors describe a plasmid which integrates through a double crossing over and therefore remains in the chromosome as a single copy.

It seems clear that all of the basic tools for genetic and molecular analyses of *Bacillus* are in hand. The unique contributions this organism can make to basic knowledge should be forthcoming.

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Gene Expression Systems for *Bacillus subtilis*

5

ANDREW MOUNTAIN

1. INTRODUCTION

The aim of this chapter is to summarize the commercial reasons for developing expression systems for making recombinant products in *B. subtilis*, to describe the problems involved in developing such systems and the approaches being taken to overcome them, and to describe briefly features of transcription, translation, and secretion that are important in the context of expression system development. The emphasis is on expression systems for production of industrial enzymes, reflecting both my main interest and my opinion that this area represents the most promising commercial application of *B. subtilis*. Although every effort has been made to keep the material up to date, it is worth pointing out that the development of expression systems is advancing very rapidly and that much of it is being performed in industry, with consequent delays in communication and publication.

2. RELATIVE ADVANTAGES OF *B. SUBTILIS* AS A HOST FOR THE PRODUCTION OF HETEROLOGOUS PROTEINS

The three main reasons for commercial interest in developing *B. subtilis* as a host for expressing the products of recombinant genes are (1) its large secretory capacity, (2) its regulatory acceptability, and (3) its well-developed fermentation and product recovery technologies (see Chapter 10). Like many strains of *Bacillus*, *B. subtilis* efficiently secretes large quantities of a variety of enzymes directly into the growth medium. Such secretion simplifies product recovery since it leaves the protein in a relatively pure, soluble form compared with an insoluble intracellular product. It also permits the accumulation of product to very high levels in an active form, unlike the denatured protein recovered from inclusion bodies which result from high-level expression and accumulation intracellularly. The downstream-processing advantages afforded by secretion are the main reason why exoenzymes of *Bacillus*, largely proteases and amylases, currently account for about 45% of the commercial enzyme market.

The long history of using *B. subtilis* for the manufacture of industrial and food products, together with its lack of pathogenicity and absence of endotoxins, has two important consequences for the future development of this organism. First, the technology for large-scale growth and product recovery is well developed and second, recombinant products from *B. subtilis* are likely to prove acceptable to regulatory authorities. The organism has already been granted GRAS (Generally Regarded As Safe) status in the United States where the Food and Drug Administration (FDA) has recently, and very significantly, given GRAS status for the first time to a heterologous enzyme (α -amylase from *B. stearothermophilus*) made in *B. subtilis* (see *Food Chemical News*, Sept. 15, 1987, p. 15).

The three main technical hurdles in developing efficient expression systems for *B. subtilis* are (1) the severe instabilities afflicting hybrid plasmids in this organism (see Chapter 7), (2) its tendency to degrade many heterologous secreted proteins, and (3) a scarcity of well-characterized gene expression signals, particularly controllable promoters. It is clear that in general eukaryotic proteins are much less efficiently secreted from *B. subtilis* but are more rapidly degraded by its proteases than are bacterial exoproteins (see Section 9).

In the short-to-medium term the most promising and feasible commercial application of *B. subtilis* lies in the production of industrial enzymes. The development of efficient expression systems promises to improve the commercial viability of a significant number of enzymes not currently available in sufficient quantities or at economically feasible prices because they originate in organisms which are pathogenic, are very difficult to grow on a large scale, or which produce the enzyme in very low yield. Alternative organisms under development as expression hosts for recombinant products in this industrial sector are primarily fungi, notably *Aspergillus* and yeast species. *Bacillus subtilis* offers more rapid fermentation and more efficient carbon conversion than these hosts and in general has a much greater secretion capacity for larger proteins (i.e., those in excess of 20 kDa) than yeasts. In addition, the development of efficient expression systems for any organism requires extensive knowledge of gene expression mechanisms at the molecular level: although *B. subtilis* obviously lags a long way behind *Escherichia coli* in this respect, its molecular biology is more advanced than that of the filamentous fungi.

3. TECHNICAL OBJECTIVES IN DEVELOPING *B. SUBTILIS* EXPRESSION SYSTEMS

There are essentially five main issues to be addressed in developing *B. subtilis* as a host for the production of heterologous proteins on a commercial scale.

3.1. Development of Systems Permitting Stable Maintenance of Cloned Genes

Manufacture of relatively low-value products like industrial enzymes involves growth of the production strain on a very large scale. The minimum realistic fer-

menter size for such an application is about 60 m³, equivalent to 60,000 liters. Growth from a single colony inoculum to high biomass (OD₆₀₀ of 50–100) on this scale requires about 60 generations. For both economic and regulatory reasons the use of antibiotics to ensure stable maintenance of the vector is undesirable. For fermentations on this scale the production strain would typically be grown through several seed stages of increasing volume leading to an inoculum into the final fermentation vessel of approximately 5%. The last 15–20 generations would need to proceed in the absence of antibiotic selection to ensure its dilution to an acceptable level. As a rough approximation a recombinant plasmid would need to show structural stability over at least 50 generations and segregational stability over at least 15–20 generations.

3.2. Development of Controllable Promoters

Strong, constitutive promoters are not generally compatible with stable vector maintenance, particularly for plasmid-based systems (Sections 5 and 6). The *spa* gene (encoding staphylococcal protein A) cannot be stably maintained in *B. subtilis* when expressed from its own powerful promoter, even when integrated into the chromosome (Saunders *et al.*, 1984a). Well-characterized promoters are required which permit stringent control over expression of the cloned gene during the growth phase of fermentation, followed by high-level expression thereafter. Where promoters are of the directly inducible type (see Section 6), it is essential, when producing low-value products, that the inducer be inexpensive.

3.3. Development of Efficient Secretion Systems

Although the regulatory status of *B. subtilis* makes it a useful organism even for the intracellular production of heterologous proteins, efficient secretion of the product into the growth medium would minimize downstream processing costs. The advantages of secretion as compared with intracellular accumulation are that it permits accumulation of the product to a very high level and in an active and relatively pure form. The main disadvantage is that the product must be recovered from a relatively dilute solution. The cost advantage of secretion therefore varies according to product yield and purification regime. Many commodity (i.e., bulk) enzymes are used with very little purification after concentration of product from the culture medium.

3.4. Construction of Protease-Defective Strains

Some exoenzymes from closely related *Bacillus* strains appear to be insensitive to the secreted proteases of *B. subtilis*, but most heterologous proteins are degraded. It has been necessary, therefore, to construct host strains which have low protease activities (see Section 9) but which nevertheless grow to a high biomass. It should be noted as well that levels of secreted proteases also vary dramatically with the composition of the growth medium.

3.5. Media and Fermentation Development

Economic production processes require the development of cheap media and fermentation conditions which permit high final biomass, rapid growth rates, relatively low protease levels, efficient and economic induction of gene expression, and high product yield from relatively short fermentations (see Chapter 10). The importance of media composition and fermentation development should not be underestimated. Historically, the development of expression systems in general (not just for *B. subtilis*) has been largely driven by advances in molecular biology and has resulted, in many cases, in systems which cannot be scaled up economically. The development of expression systems which are sufficiently robust and cost-effective for large-scale production of industrial enzymes requires interaction between fermentation technologists and molecular biologists at all stages in the process.

4. PLASMID VERSUS CHROMOSOMAL EXPRESSION SYSTEMS

The severity of instability problems affecting hybrid plasmids in *B. subtilis* has led to a great deal of interest in developing expression systems in which the gene of interest is inserted into the chromosome. The integration vectors usually consist of *E. coli* plasmids unable to replicate in *B. subtilis* but carrying a cloned fragment of *B. subtilis* chromosomal DNA to promote integrative homologous recombination and an antibiotic resistance marker for transformant selection in *B. subtilis* (e.g., Saunders *et al.*, 1984a; Janniere *et al.*, 1985; see Chapter 4). An alternative insertional system exploits the properties of the *Staphylococcus aureus* plasmid pE194, which is temperature-sensitive for replication in *B. subtilis* but which can be rescued at the nonpermissive temperature by integrating into the chromosome at a low frequency by illegitimate, homology-independent recombination (Hofmeister *et al.*, 1983; Prozorov *et al.*, 1985, 1987). Several genes which could not be established or stably maintained on conventional plasmid vectors have been stabilized by chromosomal integration (e.g., Saunders *et al.*, 1984a).

Although this approach is technically relatively simple and the cloned genes usually show a high degree of segregational stability, expression from a single copy of the gene is not necessarily satisfactory for most intended industrial applications. However, tandem amplification of such integrated genes can be readily achieved by using homologous recombination to insert a cartridge comprising the gene of interest and a suitable antibiotic resistance marker. The latter provides a means of selecting for amplification by gradual elevation of antibiotic levels (Young, 1984; Janniere *et al.*, 1985; Young and Hruanueli, 1988). Although the amplification mechanism has not yet been elucidated, it is likely to involve either unequal crossing over (Albertini and Galizzi, 1985) or spontaneous excision of the duplicated structure in circular form from one daughter chromosome and integration into the other during replication (Young, 1984). It is clear that for the homology-facilitated integrational system amplification, like integration, is *recE*-dependent (Saito *et al.*, 1983; Albertini and Galizzi, 1985). All findings published so far are consistent with an amplifiable structure comprising the antibiotic resistance marker, with or without the gene of interest, flanked by direct repeats. This structure can either be generated *in vivo* by integration of the whole transforming plasmid at a homologous

site by a single crossover (Albertini and Galizzi, 1985) or created by *in vitro* manipulations prior to integration (Janniery *et al.*, 1985; see Fig. 1). The maximum number of copies resulting from challenge with increasing concentrations of antibiotic is unpredictable, but appears to depend on the antibiotic used and the size of both the direct repeats and the whole amplified unit (Janniery *et al.*, 1985). By far the most commonly used antibiotic resistance markers are the kanamycin resistance gene of pUB110 and the chloramphenicol resistance gene of pC194. The highest copy number that can reportedly be achieved using pUB110 is 50 (Janniery *et al.*, 1985) while use of pC194 has achieved a copy number of 36 (Piggot *et al.*, 1987; Piggot and Curtis, 1987).

Although reports describing the usefulness of chromosomal amplification for expressing genes of commercial significance are scarce so far, four cloned α -amylase genes have been successfully integrated and amplified, from *B. subtilis* (Albertini and Galizzi, 1985), *B. amyloliquefaciens* (Kallio *et al.*, 1987), *B. stearothermophilus* (Diderichsen, 1986), and *B. licheniformis* (Joyet *et al.*, 1986). Up to 10-fold amplification of the *B. subtilis* α -amylase gene was achieved (Albertini and Galizzi, 1985): amplification gave a significant increase in amylase production, but the stability of the amplified state was not rigorously tested. In the case of the *B. stearothermophilus* gene a sixfold amplification was observed but details of stability and amylase levels were not reported. For the *B. licheniformis* gene 25-fold amplification was observed, but the expression level was still "extremely low," possibly

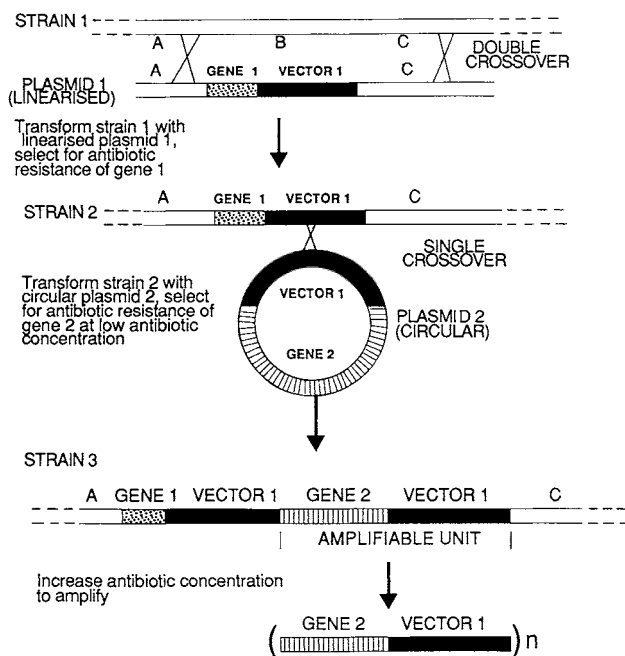


Figure 1. Insertion of genes into the *B. subtilis* chromosome in nonamplifiable form (gene 1 in strain 2) or amplifiable form (gene 2 in strain 3).

due to the inefficiency of the gene's promoter. When the amylase promoter was replaced with a more powerful one originating from the *B. subtilis* chromosome, expression increased "very significantly" but amplification was restricted to 10 copies and rendered unstable (Declerck *et al.*, 1987). The authors speculate that increasing transcription may stimulate recombination between the repeated sequences. Replacement of the amylase promoter with the inducible *B. subtilis* levansucrase gene promoter permitted sucrose induction of amylase synthesis, but 10-fold amplification of this construct led to only a two- to threefold increase in amylase production (Joyet *et al.*, 1986).

The most thorough documented study on amplified amylase genes involves that from a production strain of *B. amyloliquefaciens* (Kallio *et al.*, 1987). A *B. subtilis* strain carrying two copies of the gene integrated in different locations in the chromosome was found to be capable of producing as much amylase as a strain carrying the gene on pUB110 at 40 copies per cell. The production kinetics differed considerably, however, with amylase accumulating more slowly for the integration strain but continuing for longer. Strains carrying eight integrated copies were constructed, both by further random integration events and by a tandem amplification strategy. The kinetics of amylase production of these strains approached those of the plasmid-carrying strains but they gave greater yields of amylase than the latter, displaying a longer production phase. Although these strains showed relatively stable maintenance of the amplified genes in small fermenters, further amplification was found to be destabilizing. It is reported that both tandem and nontandem duplication have been achieved for a cloned protease gene from an alkalophilic *Bacillus* species following retransfer of the gene into the production strain (van Eikelen *et al.*, 1987). Although both types showed increased production of the protease, only the nontandem duplication showed a high level of stable maintenance.

In summary, although chromosomal integration and amplification is a promising approach to constructing recombinant production strains, few data on expression levels and stability are available as yet for cloned genes of commercial significance. It is clear, however, that at least some tandemly amplified genes are not stably maintained (Young and Hruanueli, 1988). It is reported that the frequency of recombination between duplicated sequences in the *B. subtilis* chromosome varies by more than 100-fold depending on the location of the duplication (Vagner and Ehrlich, 1986; Janniere and Ehrlich, 1987). If loss of the amplified state proceeds through homologous recombination between amplified gene copies it might be possible to reduce instability by an appropriate choice of integration site, and perhaps also by the introduction of the *recE4* allele after amplification. Preliminary experiments, however, suggest that segregation rates of amplified structures are similar in *recE4* and *recE+* strains (Young and Hruanueli, 1988).

Plasmid-based expression systems would undoubtedly be easier to manipulate than chromosomal systems providing the problems of instability can be overcome. Another obvious potential advantage of plasmid-based systems is that they should permit higher copy numbers to be achieved. It appears from the work described above that product yield from at least some cloned amylase genes is not directly related to copy number. The yield of amylase from the cloned α -amylase gene of *B. licheniformis* under the control of the *spoVG* promoter was found to be the same when integrated into the chromosome at a copy number of one, amplified in the

chromosome, or carried on a high copy number plasmid (Stephens, personal communication). In contrast, the yield of β -galactosidase from the *lacZ* gene transcribed from the *spoVG* promoter proved to be copy number-dependent. It is not clear whether the apparent independence of copy number for amylase productivity is specific to amylase or applies in general to secreted enzymes. It will be important to resolve this problem, and to identify the rate-limiting factor in amylase production (see Section 6.3).

A general problem in assessing the relative usefulness of plasmid versus chromosomal expression systems, as well as in comparing different plasmid systems, is that almost every study published has used different growth regimes for measuring segregational stability.

5. EFFECTS OF GENE EXPRESSION ON PLASMID STABILITY

As described more fully in Chapter 7, two forms of instability can afflict recombinant plasmids: structural and segregational. Structural instability refers to the deletion, acquisition, or rearrangement of sequences within it, while segregational instability refers to the loss of the whole recombinant plasmid. While both types of instability are well documented in *E. coli*, there is no doubt that both have been observed at much greater frequency in *B. subtilis*; they are discussed below largely in the context of expression systems.

5.1. Structural Instability

Whatever the mechanism(s) of deletion formation, it is clear that a large proportion of deletions occurring during or after plasmid establishment are associated with uncontrolled transcription of the cloned DNA. In some cases the consequences of transcription are so deleterious, either for the cell or for the plasmid, that transformants carrying the intact hybrid are never recovered (e.g., Saunders *et al.*, 1984a,b). Strong promoter activity can lead to the selection of deleted forms in a number of different ways. First, such activity may interfere with one or more essential plasmid functions, e.g., transcriptional readthrough into the region of the plasmid responsible for copy number control can result in a decrease in copy number or even prevent its replication altogether. In the absence of antibiotic selection this is likely to lead to segregational instability (see Section 5.3). Growth in the presence of relatively high levels of antibiotic may select for deletions which remove the promoter (Saunders *et al.*, 1984b; Lopez *et al.*, 1984). This type of instability may be prevented by protection of the replication functions with transcription terminators, both in *E. coli* (Gentz *et al.*, 1981) and in *B. subtilis* (Kupsch, 1986). A second consequence of strong promoter activity may be high-level expression of genes with products which are detrimental to the cell, most often of genes also located within the insert DNA but sometimes of antibiotic resistance genes (particularly tetracycline-resistance) or other vector sequences. This leads to selection for deletions which remove either the promoter or all or part of the deleterious gene. Preliminary evidence suggests that this situation arises when attempts are made to express at least some genes encoding secreted products from

promoters which are highly active in the growth phase (Nugent and Mountain, unpublished; see Section 8). Until recently, vectors with suitable transcription terminators were not readily available to enable the distinction between these two causes of selection for deleted forms. It is also conceivable that strong promoter activity leads not only to selection for deleted forms but also to an increase in the frequency of deletion formation: local unwinding during transcription might render the DNA more susceptible to recognition by enzymes involved in illegitimate recombination.

It should be emphasized here that the presence of direct repeats, topoisomerase recognition sites, or particular DNA secondary structures is not the actual cause of deletions in hybrid plasmids; these features merely permit the cell's various recombination systems to generate deletions. *recE*-independent deletion events probably occur constantly in many hybrid plasmids at a relatively low frequency. Whether or not the deletions are observed and the time it takes for them to predominate depends largely on the effects of the insert on replication and stability functions of the plasmid, and on the generation time for the cell. A pUB110 hybrid carrying an α -amylase gene, for example, proved much less deletion-prone in cells grown in the presence rather than the absence of glucose, which exerts catabolite repression on amylase synthesis (Vehmaanpera and Korhola, 1986). The deletions in this and at least one other case (Harrington *et al.*, 1984) were not observed until late stationary phase, which coincides with the main production phase for amylase. Where the insert DNA leads to high-level expression of a deleterious product or interferes with essential plasmid functions, deleted forms will either predominate very rapidly or it may even prove impossible to recover transformants containing the intact hybrid (Saunders *et al.*, 1984a,b). If the insert leads to high-level expression of a gene with a nontoxic product, metabolic load effects can exert selective pressure in favor of deletion derivatives. Even in cases where the insert contains neither a strong promoter nor a gene with a detrimental product, cells carrying deleted forms may still have a significant growth advantage if the insert lies in a position in the plasmid which leads to the accumulation of single-stranded or gapped concatameric replication intermediates, since these appear to adversely affect the cell's physiology (Bron *et al.*, 1987b; Viret and Alonso, 1987). Conversely, deleted forms may never be observed if cells containing them have no selective advantage: the observation that no deletions were observed in certain cointegrates of pE194 and pUB110 despite their carrying 22 base pairs or 29-base pair direct repeats may be an illustration of this point (Hahn and Dubnau, 1985).

5.2. Minimizing Structural Instability

Now that transcription terminators have been identified which function efficiently in *B. subtilis*, e.g., the coliphage λ c⁰ and *E. coli* *rrnB* T1 and T2 terminators (see Section 6.4), it is possible to minimize deletions resulting from the presence of strong promoters in the insert DNA by using vectors which have transcription terminators positioned and oriented to prevent readthrough into the replication region(s) of the plasmid. This is particularly important when shotgun-cloning fragments from organisms with DNA of high AT content. Most of the entire genome of *Streptococcus pneumoniae* proved unclonable in high copy number vectors of *E. coli* without transcription terminators for this reason (Chen and Morrison, 1987).

For shotgun cloning of genes with secreted products it is advisable not to use expression plasmids which provide strong constitutive promoters because it appears likely that overloading the cell's secretory capacity for a particular protein leads rapidly to the selection of deletion derivatives. The use of promoters which are inactive or relatively weak during the growth phase is recommended, e.g., those of amylase or protease genes (see Section 6). In general, when cloning into *B. subtilis* for expression it is advisable to use expression vectors which feature controllable promoters analogous to the *lac*, *tac*, and λ pL promoters which have proven so useful for cloning in *E. coli*. Such promoters are now becoming available (see Section 6).

There are many cases of genes which can be cloned in low copy number vectors in *E. coli* which had previously proved unclonable or impossible to maintain intact in high copy number vectors (Mountain *et al.*, 1984) due to the presumed deleterious effects of the gene product on the cell. Low copy number vectors are now becoming available for cloning in *B. subtilis* (Imanaka *et al.*, 1986; Bron *et al.*, 1987a).

5.3. Segregational Instability

Uncontrolled transcription from within the insert DNA can affect segregational stability in two main ways: first, transcriptional readthrough into replication functions of the plasmid can reduce its copy number below that required for stable inheritance at cell division; second, high-level gene expression during the growth phase can lead to a selective advantage for plasmid-free cells through increased metabolic load. In extreme cases the growth rate of plasmid-bearing cells may be affected to a degree which itself impairs plasmid replication (Aiba *et al.*, 1982). These problems may be circumvented by using efficient transcription terminators and tightly controllable promoters, neither of which was available for *B. subtilis* until very recently.

Although no strict experimental comparisons have ever been made, it appears from the literature that pUB110 hybrids are maintained somewhat more stably in *B. subtilis* than those derived from other *S. aureus* replicons. Partly for this reason and partly because of its greater copy number (40–50 per cell), almost all attempts to construct expression plasmids for this organism have used the pUB110 replicon. The parental replicon is maintained with 100% stability over 100 generations in a variety of test strains (Mountain, unpublished) but it is destabilized to varying extents by the presence of insert DNA. One thorough study (Bron and Luxen, 1985) revealed a steep inverse relationship between insert size and stability for pUB110-derived vectors. In rapidly growing cultures an inverse relationship was also found between insert size and plasmid copy number, presumably due to partial impairment of the copy number control mechanism. It appears likely that pUB110 is analogous to colE1-derived vectors in *E. coli*, in that it is a high copy number plasmid which probably lacks an active partitioning function and segregates randomly into daughter cells at cell division. Any manipulations which reduce its copy number, such as the insertion of foreign DNA, will increase the frequency with which plasmid-free cells arise. The pUB110-derived vectors in these studies lacked two membrane-binding regions, BA3 and BA4 (see Chapter 7), which may be involved in plasmid replication (McKenzie *et al.*, 1986). Deletion of BA3 is reported to lead to the accumulation of single-stranded replication intermediates which are

deleterious to cell physiology (Viret and Alonso, 1987). For these vectors, then, even without insert DNA there is probably a significant selective advantage for plasmid-free cells. Manipulations of the plasmid that result in an increase in single-stranded intermediates may thus increase segregational instability as well as structural instability. Recent evidence indicates that other manipulations may lead to the accumulation of gapped concatameric intermediates with similar effects (Viret and Alonso, 1987). Studies on the stability of hybrids comprising inserts of various sizes cloned into intact pUB110 (Bron *et al.*, 1987b) suggested that the onset of plasmid loss is delayed by about 20 generations compared to those lacking BA3 and BA4. For unknown reasons some pUB110 hybrids appear to show more stable maintenance in *recE4* strains than in *recE⁺* strains (Reid *et al.*, 1986).

5.4. Strategies for Developing Stable Expression Vectors

As outlined in Section 3.1, a very high level of stable maintenance in the absence of antibiotic selection is a primary objective in developing expression plasmids for large-scale production. Considering our limited understanding of even the most frequently used plasmid replicon in *B. subtilis* (i.e., pUB110), it is advisable to minimize disturbances to the plasmid replication and stability functions by avoiding unnecessary additions or deletions of DNA. Such precautions have not generally been taken into account when constructing expression plasmids from pUB110. Most such plasmids are in fact shuttle vectors and therefore carry several kilobases of extra DNA which are required for replication, maintenance, and transformant selection in *E. coli*. Many such vectors have been made which lack pUB110 sequences of unknown function in order to simplify construction. Similarly, the choice of restriction sites for cloning promoters and structural genes has generally been based purely on the convenience of construction. A systematic study is required to identify cloning sites in pUB110 where the presence of insert DNA has minimal effects on replication and stability. Such a study is now under way and has already revealed that hybrids with a 5-kilobase insert in the *Bam*HI site suffer both structural and segregational instabilities at a much higher frequency than those with the insert in the *Bgl*II site (Gleave, personal communication). Once the optimal cloning site has been identified, inserted DNA should be on the smallest possible fragment and should be flanked by transcription terminators.

Probably the most stable published pUB110 hybrid of any commercial significance is pKTH10 (Palva, 1982), which carries the α -amylase gene from *B. amyloliquefaciens* cloned on a 2.3-kilobase partial *Sau*3A fragment. This plasmid has proved to be stable enough for batch production of the α -amylase (Vehmaanpera and Korhola, 1986; Vehmaanpera *et al.*, 1987). The relatively stable maintenance of this plasmid may be due to the relatively small size of the insert, the lack of deletions in pUB110 DNA, and the presence of transcription terminators found to flank the amylase gene (Lehtovaara *et al.*, 1984). Studies with pKTH10 illustrate also the importance of the properties and activity of the promoter from which the cloned gene is expressed. In normal host strains the amylase promoter is relatively inactive during the growth phase, and consequently imposes little metabolic load on the cells at this time. The promoter is more active in stationary phase, which is the main amylase production phase, leading to slight plasmid loss during this time with 3.5% of cells plasmid-free after 12 hr in stationary phase (Vehmaanpera *et al.*, 1987). In a

strain carrying the *sacU9* mutation, which leads to increased expression from the amylase promoter (see Section 6), only 0.1% of cells retained pKTH10 after 50 generations in a serial subculture experiment compared to 100% for normal strains (Vehmaanpera and Korhola, 1986). Growth of the *sacU9* strain in the presence of glucose, which represses amylase synthesis about fourfold, resulted in 99.5% of the cells retaining the plasmid. These experiment provide a powerful demonstration of the need for tightly controlled promoters to reduce metabolic load during the growth phase.

A number of different approaches are being taken in attempts to stabilize pUB110 hybrids sufficiently to permit their use in large-scale industrial fermentations. One approach is to use positive nutritional selection, in which growth of the host strain is made dependent on the product of a gene carried on the expression plasmid. The most promising such system employs the *B. subtilis dal* gene, which encodes D,L-alanine racemase. This enzyme is required for the provision of D-alanine, a constituent of cell wall peptidoglycan, even during growth in rich media. A pUB110 hybrid carrying the *dal* gene and a cloned *B. stearrowthermophilus* α -amylase gene was found to be maintained with 100% stability over 85 generations in a strain with a chromosomal *dal* deletion (Diderichsen, 1986). A similar *dal* system has been developed which permitted 100% stable maintenance of another pUB110 derivative (Ferrari, E. *et al.*, 1985). A possible drawback of such positive selection systems is loss of the plasmid through integration of the selective marker or the entire plasmid into the chromosome, since a single copy of the *dal* gene will suffice for cell growth.

A second approach involves cloning into pUB110 hybrids active partitioning functions from endogenous, low copy number *B. subtilis* plasmids. Two such studies have identified a stability "*stab*" locus in the plasmid pTA1060 (Bron *et al.*, 1987a) and a partitioning or *par* function in the plasmid pLS11 (Chang *et al.*, 1987). It is now apparent that these two plasmids are identical, as are the *par* and *stab* loci (S. Bron, personal communication). In one study (Chang *et al.*, 1987) a fragment containing *par/stab* was found to confer a high degree of segregational stability on several highly unstable shuttle vectors derived from pUB110 and other replicons. In the other study a different fragment carrying *par/stab* was shown to delay significantly the loss of an unstable pUB110-derived shuttle vector (Bron *et al.*, 1987a). The effectiveness of this approach for stabilizing hybrids carrying highly expressed genes of commercial significance remains to be determined, particularly since in the analogous situation in *E. coli* the pSC101 *par* function is not capable of conferring stable maintenance on all pBR322- or pACYC184-derived hybrids (Skogman *et al.*, 1983).

Another elegant general approach for ensuring plasmid retention would be to arrange for the death of plasmid-free segregants. A system is under development which aims to exploit the *parB* system of the *E. coli* plasmid R1. *parB* consists of two genes: *hok*, responsible for killing plasmid-free cells, and *sok*, a suppressor of *hok* (Gerdes *et al.*, 1986). Expression of the *hok* gene from an inducible promoter has been shown to be toxic in *B. subtilis* (Poulsen and Andersson, 1986). It may therefore be possible to develop a stable maintenance system which would involve carriage of *sok* on the expression plasmid in a host strain with *hok* in the chromosome.

One of the main reasons for the segregational instability of pUB110 hybrids appears to be that most inserts reduce its copy number (Bron and Luxen, 1985).

One approach to reducing segregation rates is therefore to increase plasmid copy number. For example, chromosomal mutants selected for increased kanamycin resistance have been isolated in which pUB110 and derivatives show elevated copy number. A pUB110 hybrid carrying the *B. subtilis* α -amylase gene was found to be maintained with 100% stability after 55 generations without selection in such an "IRK" strain (Lejeune, 1985). In a wild-type strain only 3% of cells retained the plasmid under these circumstances.

Very stable maintenance can only be achieved if plasmid replication, cell division coupled to replication, and partitioning of plasmids into daughter cells are all carried out efficiently. Considering our knowledge of the mechanisms of these processes in *E. coli* and the specificity of DNA/protein interactions in general, the most realistic chance of achieving stable plasmid maintenance in *B. subtilis* lies in developing expression vectors from endogenous *B. subtilis* plasmids with active partitioning functions. Such plasmids might be expected to have evolved for stable maintenance in this organism and to have proliferation properties compatible with it. This approach offers the additional advantage that sequences which encourage deletion formation through illegitimate recombination in *B. subtilis* may have been removed during its long-term residence in this host.

A series of vectors derived from an endogenous *B. subtilis* plasmid was developed in my laboratory. The starting plasmid, pPOD2000, is 8.5 kilobases in size and maintains a copy number of 10 per cell in all growth phases. This cryptic plasmid has been converted into cloning vectors by random insertion of a genetic labeling cassette comprising a chloramphenicol resistance gene for transformant selection, unique restriction sites to facilitate cloning, and an efficient transcription terminator to prevent plasmid destabilization by readthrough transcription from strong promoters cloned into those sites. Segregational stability tests on the resulting cassette insertants identified several showing normal copy number and completely stable maintenance. These stable vectors have been shown to maintain a variety of inserts with unchanged copy number and 100% stability over at least 80 generations, including an α -amylase gene expressing from its own promoter which dramatically destabilizes pUB110 (Mountain *et al.*, unpublished). Similarly, the low copy number plasmid (5 per cell) pTA1060 has been shown to maintain stably a chloramphenicol/erythromycin resistance fragment with an unchanged copy number for 100 generations (Bron *et al.*, 1987a).

Although the relatively low copy numbers of these vectors may be regarded as a disadvantage for achieving very high levels of gene expression, the pPOD2000-amylase hybrid permits accumulation of amylase secreted into the medium to 2.5 g/liter (Mountain *et al.*, unpublished). It may be possible to improve this yield by replacing the amylase promoter with a stronger, controllable one and perhaps also by incorporating a copy number amplification system. Experiences in the development of *E. coli* expression systems suggest that copy number amplification may be a fruitful approach to developing stable, high-level expression systems. One such *E. coli* system permits amplification of copy number from four per cell in the growth phase to about 200 per cell in the production phase (Caulcott *et al.*, 1985). In this system expression of the target gene from the *trp* promoter is tightly controlled at the lower copy number by a single chromosomal *trpR* gene. The plasmid therefore imposes very low metabolic load and shows very stable maintenance through the growth phase. Copy number amplification, achieved by thermoinduction at the

start of the production phase, derepresses the *trp* promoter by repressor titration and gives very high-level expression of the target gene (typically to 5–10% of total cell protein). A primitive thermoinducible copy number amplification system has already been constructed which permits a fivefold increase in the copy number of pPOD2000 derivatives in *B. subtilis* (Nugent and Mountain, unpublished).

6. TRANSCRIPTION

6.1. Multiple σ Factors

The RNA polymerase of *B. subtilis* is a remarkably heterogeneous enzyme which is found in many different holoenzyme forms in both growing and sporulating cells (Losick and Pero, 1981). Each holoenzyme form contains a particular species of σ factor which confers on the core enzyme (E) the ability to recognize and utilize a distinct family of promoters (see Chapter 8). Although more attention has recently been given to the role of σ factors in sporulation, it is clear that some *B. subtilis* phages exploit a similar mechanism to achieve sequential gene expression (Geiduschek and Ito, 1982 and references therein). The lytic phages SP01 and SP82 encode three classes of transcript: early, middle, and late. Early transcripts are made by host RNA polymerase, whereas phage-specified proteins are required to produce middle and late transcripts. For phage SP01 transcription of middle genes requires the expression of phage gene 28, and that of late genes expression of phage genes 33 and 34. The product of early gene 28 is a σ factor of 26 kDa and is able to confer the ability to utilize middle-gene promoters on core polymerase (Costanzo and Pero, 1984). Genes 33 and 34 are middle genes encoding proteins of approximately 12 and 24 kDa, respectively. Specific production of late transcripts *in vitro* requires association with core polymerase of both these phage proteins and another host protein, the δ factor (Tijan and Pero, 1976). SP82 encodes proteins analogous in size and probably also in function to the SP01 gene 28, 33, and 34 products (Achberger and Whitely, 1980). The lytic phage ϕ 29 encodes only two temporal classes of transcript, early and late. The phage gene 4 product is required for late transcripts to appear *in vivo*. Partially purified gene 4 product confers on core polymerase the ability to produce two late transcripts *in vitro*, consistent with the idea that gene 4 encodes a σ factor (Mellado *et al.*, 1986).

It now appears that the exploitation of alternative σ factors as a mechanism for regulating the expression of discreet sets of genes is not confined to temporal regulation during the irreversible processes of sporulation and lytic phage development. Recent evidence indicates that alternative σ factors are also involved in transient responses to environmental stress, such as heat shock and nitrogen limitation in enteric bacteria (see Doi and Wang, 1986). It is not yet clear how use of these σ factors is controlled or how they are displaced by phage-specified forms. Possible mechanisms are discussed in Reznikoff *et al.* (1985).

For comprehensive surveys of the evidence for multiple σ factors and current ideas on their roles in promoter selectivity, the reader is referred to two excellent recent reviews by Reznikoff *et al.* (1985) and Doi and Wang (1986). Amino acid sequence homologies among the σ factors found in *E. coli* and *B. subtilis* cells and

their implications for interactions with core polymerase and promoters were also recently reviewed (Gribkov and Burgess, 1986).

6.2. σ Factors and Promoter Recognition

The exact molecular basis of promoter selectivity by σ factors is not yet clear. It is generally accepted that prokaryotic promoters (except some specialized phage promoters) contain two regions, centered around -35 and -10 with respect to the $5'$ end of the messenger RNA, important for RNA polymerase binding. There is usually sequence conservation at these two regions among promoters utilized by the same σ factor. The most favored model of promoter recognition suggests that each σ factor recognizes sequentially its cognate -35 region during closed complex formation, and then its cognate -10 region before forming the open initiation complex (Losick and Pero, 1981). A recent alternative model (Cowing *et al.*, 1985) proposes that each σ factor interacts directly only with its cognate -10 region, with -35 region contacts being made by core enzyme subunits. It is now technically possible to test these models by introducing site-directed changes into promoters and into sequences coding for particular σ factors.

It has been noted that a large proportion of the operons of *Bacillus* that have been studied in molecular detail appear to be transcribed from complex promoter regions (Doi and Wang, 1986). These regions usually take the form of tandem or overlapping promoters recognized by different σ factors and presumably allowing expression in a variety of different nutritional conditions or stages of development. Transcription from multiple promoters may indeed prove to be a common feature of *Bacillus*, but it equally may reflect the emphasis on studying genes involved in differentiation for these organisms.

6.3. σ^{43} and Transcription Initiation

In the context of the development of expression systems, there is considerable interest in determining the factors which contribute to promoter efficiency (or promoter "strength"). Factors suggested (Moran *et al.*, 1982) to be involved in determining the efficiency of promoters recognized by σ^{43} include the degree of homology to the -35 and -10 consensus sequences; the spacing between the -35 and -10 regions; the AT content between -35 and -50 ; the AT content between sequences -6 and $+1$; and the degree of homology to a third possible consensus sequence, PuTPuTG, centered around -16 .

The consensus -35 and -10 sequences recognized by the major vegetative σ factors of *E. coli* and *B. subtilis* are identical. It is not therefore surprising that many *B. subtilis* genes with promoters recognized by σ^{43} are efficiently transcribed by *E. coli* RNA polymerase, both *in vitro* (Moran *et al.*, 1982) and *in vivo*. It appears, however, that only a small fraction of *E. coli* promoters are utilized efficiently *in vivo* by $E\sigma^{43}$ (Wiggs *et al.*, 1979; Lee *et al.*, 1980; Zukowski *et al.*, 1983). To account for this discrepancy it has been suggested that $E\sigma^{43}$, unlike $E\sigma^{70}$ of *E. coli*, may require a very specific length for the spacing between -35 and -10 (Reznikoff *et al.*, 1985) and also a highly AT-rich region immediately upstream of the -35 sequence (Moran *et al.*, 1982) for efficient initiation. The ability of $E\sigma^{43}$ to utilize several powerful

and well-characterized *E. coli* promoters has been thoroughly studied both *in vitro* by runoff transcription and *in vivo* using a promoter-probe vector in which the promoters were fused to *cat* as the reporter gene (Peschke *et al.*, 1985). The *tac*, phage T7A1, phage T5 PN26, and PJ5 promoters were all utilized efficiently both *in vivo* and *in vitro*. The PN26 promoter proved most efficient: when fused to *cat* on a multicopy plasmid, both PN26 and *veg*—one of the strongest known *B. subtilis* promoter complexes—gave CAT levels comprising approximately 25% of total cell protein. The spacings for the *tac*, PN26, and *vegII* promoters are 16, 17, and 18 base pairs, respectively, arguing against a very rigid spacing requirement for efficient promoter utilization by $E\sigma^{43}$. Consistent with this view is the recent finding (Henkin and Sonenshein, 1987) that reducing from 18 to 17 base pairs the spacing in a series of promoters derived from the *E. coli lacUV5* promoter had only slight effects on promoter strength. Two promoters believed to be utilized by $E\sigma^{43}$ have spacings of 22 and 21 base pairs. These are, respectively, the promoters of the *sigE* gene (encoding σ^E) and the *spoIIIE* gene for which additional protein factors interacting with symmetrical DNA sequences may be required to overcome the unusually long spacing (Kenney *et al.*, 1987; Guzman *et al.*, 1987).

Promoters found to be utilized very efficiently *in vivo* (Peschke *et al.*, 1985) are extremely AT-rich (80–90%) between sequences –35 and –50; however, the fairly efficient *tac* promoter is only 60% AT-rich in this region. T5PN26, T5PJ5, and T7A1 are all 80% AT-rich in this region and have strikingly different efficiencies. Deletions which drastically reduce the AT content upstream of –45 in the *veg* promoter were found to have only slight effects on its efficiency (Le Grice *et al.*, 1986). In contrast, similar deletions in the *S. pneumoniae mal* promoters dramatically reduce their efficiency in *B. subtilis* (Espinosa *et al.*, 1984). Similar deletions in the *spoVG* promoter, which is utilized by both $E\sigma^B$ and $E\sigma^C$, drastically reduce its efficiency (Banner *et al.*, 1983). All the promoters so far described which are utilized very efficiently *in vivo* by $E\sigma^{43}$ (T5PN26, *veg*, SP01P15, and P26, and the ribosomal RNA promoters) are extremely AT-rich between –35 and –50. There is evidence that high-level expression in *E. coli* from the powerful λ pL and *trp* promoters is similarly dependent on upstream AT-rich blocks (Horn and Wells, 1981; Nishi and Ito, 1986). Replacement of two GC base pairs interrupting an AT-rich stretch between –35 and –55 in the fairly weak promoter of the *B. amyloliquefaciens* α -amylase gene, however, was found to reduce its efficiency, at least in *B. subtilis* (Mizrahi *et al.*, 1987).

The T5PN26 promoter has perfect homology with the consensus promoter only in its –10 region, and T5PJ5 only in its –35 region, while T7A1 differs in both but is more efficient *in vivo* than PJ5. All three of these phage promoters are utilized more efficiently *in vivo* than the *tac* promoter, which conforms to the consensus sequences at both –35 and –10. The very efficient *B. subtilis veg* promoter complex comprises two neighboring RNA polymerase binding sites, termed sites I and II (Le Grice *et al.*, 1986). Site I has a consensus –35 and one difference from the consensus –10, with 17 base pairs spacing. Only site I normally produces a transcript, though the upstream site II has perfect consensus –35 and –10 regions with 18 base pairs spacing. Insertion of a linker between sites I and II allowed site II to produce a transcript, an effect attributed to placing a purine residue 7 base pairs 3' to the –10 region.

The *E. coli lacUV5* promoter is utilized only weakly *in vivo* by $E\sigma^{43}$, despite

having 5/6 and 6/6 identifies with the consensus -35 and -10 regions, respectively (Lee *et al.*, 1980). In a recent study (Henkin and Sonenshein, 1987), random mutations conferring increased activity on this promoter in *B. subtilis* were characterized and were found to fall in two clusters. One set of mutations resulted in greater homology to a consensus sequence, PuTPuTG, centered around -16 and previously noted for this reason in several vegetative *B. subtilis* promoters (Moran *et al.*, 1982). The remaining mutations were clustered around the transcription initiation site and either increased the number of A residues between -6 and -2 on the nontemplate strand, or changed the +1 or +2 residues from A to G. Dramatic increases in promoter strength were achieved by converting the -16 and -35 sequences to the consensus ones by oligonucleotide-directed mutagenesis. Seemingly at odds with some of these findings is the fact that many heterologous promoters that function efficiently in *B. subtilis* have little homology with the conserved -16 region and are not A-rich between bases -6 and -2.

Rather few promoters utilized by $E\sigma^{43}$ have been analysed by high-resolution S1 mapping (or equivalent techniques) to determine the precise starting point of transcription at the nucleotide level. Three *B. subtilis* promoters which have been studied in detail are the major promoters of the *gnt* (Fujita and Fujita, 1986), *trp* (Shimotsu *et al.*, 1986), and *arg* (Smith *et al.*, 1986) operons. The preferred mRNA 5' ends in these cases are A's or G's located 6-8 base pairs downstream from the 3' end of the -10 sequence, results very similar to those for *E. coli* promoters utilized by $E\sigma^{70}$. In contrast, the α -amylase promoter of *B. amyloliquefaciens*, which is believed to be utilized by $E\sigma^{43}$ in *B. subtilis*, produces transcripts initiating at two consecutive U residues with spacings of only 4 and 5 bases from the -10 region (Lehtovaara *et al.*, 1984). In thorough studies on *B. subtilis* phage ϕ 29 (Mellado *et al.*, 1986), precise S1 mapping was performed on six early promoters believed to be utilized by the host $E\sigma^{43}$: transcripts were found initiating with all four dNTPs and with spacings of between 1 and 6 bases. It appears, therefore, that the spacing between the -10 region and the initiating dNTP are much more variable for $E\sigma^{43}$ in *B. subtilis* than for $E\sigma^{70}$ in *E. coli*.

It is clear from the somewhat conflicting findings described above that a multitude of factors are involved in determining the efficiency with which $E\sigma^{43}$ utilizes its cognate promoters, and the relative importance of these factors has yet to be clearly defined. This is not surprising, since there is ample evidence that the sequences of *E. coli* promoters encode complex functional pathways which evolution has optimized in several differing ways (Brunner and Bujard, 1987). One of the recent studies referred to above (Henkin and Sonenshein, 1987) illustrates that structure-function studies on promoters should not be confined to the well-known conserved regions. It is important to point out also that analysis of promoter strength *in vivo* can give results different to those of *in vitro* methods (Brunner and Bujard, 1987). The only conclusions that can be drawn at this stage are that promoters which are utilized very efficiently by $E\sigma^{43}$ usually conform to the consensus sequences TTGA— at -35 and TA—AT at -10 and have a highly AT-rich region between -35 and -50. The spacing between -35 and -10 for such very strong promoters is usually 17 or 18 base pairs, but there is clearly no absolutely rigid requirement for this spacing as was once suggested by Moran *et al.* (1982). There is evidence that in *E. coli* utilization of some promoters by $E\sigma^{70}$ is influenced by DNA supercoiling (see Reznikoff *et al.*, 1985) and DNA bending (Galas *et al.*, 1985;

Plaskon and Wartell, 1987). The influence of DNA topology and conformation on promoter activity in *B. subtilis* is at present unknown.

6.4. Choice of Promoters for Expression Systems

The available evidence indicates that the transcription machinery of *B. subtilis* is capable of utilizing most promoter sequences originating in other *Bacillus* species, but only a small minority of those from *E. coli* and other gram-negative bacteria. For example, shotgun cloning into promoter-probe plasmid pTG402 of chromosomal fragments from *B. subtilis*, *B. pumilus*, and *B. licheniformis* (Zukowski *et al.*, 1983) gave very similar frequencies of clones in which the *xylE* reporter gene was activated in *B. subtilis*, whereas *E. coli* chromosomal fragments gave a frequency at least 20-fold lower. No phage λ fragments displayed promoter activity. Promoters which have been used or are now being used in the development of expression plasmids include those from the following sources: *B. subtilis* phages SP01 (Yansura and Henner, 1984a; Osburne and Craig, 1986), SP02 (Williams *et al.*, 1981), ϕ 105 (Dhaese *et al.*, 1984; Osburne *et al.*, 1985), coliphage T5 (Peschke *et al.*, 1985), the *erm* gene of pE194 (Hardy *et al.*, 1981; Grandi *et al.*, 1985), the penicillinase gene (*penP*) of *B. licheniformis* (Chang *et al.*, 1982; Himeno *et al.*, 1986), the α -amylase genes of *B. amyloliquefaciens* (Ulmanen *et al.*, 1985; Fahnestock and Fisher, 1986; Schein *et al.*, 1986) and *B. subtilis* (Shiroza *et al.*, 1985; Yamane *et al.*, 1986), protease genes of *B. amyloliquefaciens* (Vasantha and Thompson, 1986; Honjo *et al.*, 1986) and *B. subtilis* (Doi *et al.*, 1986; Zukowski and Miller, 1986), and the *B. subtilis* levansucrase gene Joyet *et al.*, 1986; Zukowski and Miller, 1986). Reports are now beginning to emerge of the use of synthetic promoters, either with consensus -35 and -10 regions or based on sequences of some of the promoters listed above (e.g., Zukowski and Miller, 1986).

To be of value for the production of heterologous products in large-scale fermentations, promoters must display two properties: their activity should be compatible with stable maintenance of the cloned gene in the absence of antibiotic selection through the biomass accumulation phase of the fermentation, and they should permit accumulation of the cloned gene product to high levels at the productive stage of the fermentation. High-level expression of plasmid-borne cloned genes during the growth phase gives plasmid-containing cells a growth rate disadvantage, leading to segregational instability of the recombinant plasmid. Expression plasmids which carry strong unregulated promoters such as those from phages T5, SP01, and SP02, although useful for laboratory scale experiments, are not suitable for industrial scale production.

One of the main problems which has delayed the development of stable, efficient *B. subtilis* expression systems is the lack of well-characterized, strong controllable promoters analogous to the λ pL, λ pR, *trp*, *lac*, and *tac* promoters which have proven so useful in *E. coli*. Basically, two types of promoter system are being developed for *Bacillus*. The first involves the use of promoters which naturally show growth phase-dependent expression, such as those of most *Bacillus* α -amylase and protease genes. These promoters are relatively inactive during the growth phase but are activated during the onset of stationary phase, and continue to be active for many hours in stationary phase. The molecular basis for this temporal control is in almost all cases not well understood. Some but not all of these promoters may be

recognized by minor σ factors. The promoters for the α -amylase genes of both *B. subtilis* (Nicholson and Chambliss, 1985; Nicholson *et al.*, 1987) and *B. amyloliquefaciens* (Lehtovaara *et al.*, 1985) are utilized by $E\sigma^{43}$. Transcription of the alkaline protease (*apr*) gene of *B. subtilis* has been shown to be unaffected in strains lacking both σ^C and σ^F and it is believed to be transcribed by $E\sigma^{43}$ (Ferrari *et al.*, 1987). In some cases postexponential activation may reflect release from catabolite repression, but this is apparently not the case for the *B. subtilis* α -amylase promoter since mutations which confer insensitivity to glucose cause no change in temporal regulation. In the case of the *B. amyloliquefaciens* α -amylase promoter, glucose repression appears to be exerted posttranscriptionally (Yoshikawa *et al.*, 1987). One possible explanation for temporal regulation of these promoters is that their efficient utilization requires the presence of one or more positively acting factors which are not synthesized until the end of the growth phase. These factors are likely to include the products of one or more of the *sacU*, *sacQ*, and *priR* genes. A requirement for these factors might also explain why expression levels for amylase are apparently almost independent of copy number: expression from such promoters would be limited by the number of molecules of these factors in the cell.

One particular advantage of using amylase and protease promoters to express secreted products is that single fragments can be employed to provide a promoter, translation initiation region, and signal sequence, all of which have evolved to be compatible with each other and with efficient secretion (see Section 8). Such promoters, however, tend to be relatively weak, so that accumulation of cloned gene products to high levels requires prolonged stationary phase fermentation. This leads to problems of long and therefore relatively expensive fermentations, a tendency for the cells to sporulate or undergo autolysis, and exposure of secreted products to maximal levels of extracellular proteases. Nevertheless, the use of some of these promoters on high copy number plasmids has permitted the accumulation of amylases, proteases, and staphylococcal protein A to about 3 g/liter (Palva *et al.*, 1981; Vasantha and Thompson, 1986; Fahnestock and Fisher, 1987).

A common strategy, albeit with many variations, is being explored by several groups in attempts to both increase the yield and shorten fermentation times for expression/secretion systems. This strategy involves the overproduction of positively acting factors which exert a global control on several genes coding for secreted enzymes. These factors include the products of the *sacU* (Aubert *et al.*, 1985), *sacQ* (Yang *et al.*, 1986), *priR* (Tanaka *et al.*, 1987), and *sen* (Wang, L. F., *et al.*, 1987) genes. Table I summarizes the information currently available on the enzymes whose synthesis is affected by these factors, as well as by the product of the *hpr* gene which probably exerts negative regulation on various protease genes (Henner *et al.*, 1987). The mechanisms involved in these global regulatory circuits are not well understood, but increased levels of secreted enzymes have been shown to be correlated with overproduction of the *sacQ* and *priR* gene products, which are 46 and 60 amino acids in length, respectively (Henner *et al.*, 1987). These proteins have no significant homology to any other proteins and appear to be too small to have both sensory and DNA-binding functions. They may therefore be intermediates in the regulatory systems. It has been suggested that they are transcribed by $E\sigma^D$ (Henner *et al.*, 1987). Increased levels of secreted enzymes are also correlated with overproduction of the regulator in the case of *sacU* mutants (Aubert *et al.*, 1985). The *sacU* product is a 46-kDa polypeptide and cells overproducing it show increased

Table I. Global Regulatory Circuits Controlling Expression of Polymer-Degrading Enzymes in *Bacillus subtilis*^a

Target gene	Enzyme	<i>hpr</i>	<i>sacU</i> (Hy)	<i>sacQ</i> (Hy)	<i>prtR</i>
<i>sacB</i>	Levansucrase	—	+	+	+
<i>nprE</i>	Neutral protease	+	+	+	+
<i>aprA</i>	Alkaline protease	+	+	+	+
<i>xylA</i>	Xylanase	nd	nd	+	nd
<i>bgl</i>	β -Glucanase	nd	+	+	nd
<i>isp</i>	Intracellular serine protease	+	+	+	nd

^and, not determined. +, —, Expression of α -amylase (encoded by *amyE*) is elevated by *sacU*(Hy) and *sacQ*(Hy), but only in minimal medium with starch as sole carbon source. +, level of enzyme elevated by the mutation indicated; —, level of enzyme unaffected.

amounts of a membrane polypeptide which is the same size but has not been proven identical. In the case of *hpr*, overproduction of proteases is correlated with deletion of the regulator (Henner *et al.*, 1987). The *sacU*, *sacQ*, and *prtR* products have all been shown to exert their effects at the transcriptional level, increasing steady-state levels of mRNA for levansucrase and subtilisin but not altering the transcription start site. Deletion analysis on the levansucrase and subtilisin promoters suggests that the *sacU*, *sacQ*, and *prtR* products interact with sequences between –100 and –150, and the *hpr* product with those between –200 and –400 (Henner *et al.*, 1987). These “upstream activating sequences” are much more distant from the transcription initiation site than almost all other such regulatory or activating regions in prokaryotes, the most notable exception being the *nifA*-binding site in nitrogen-regulated genes of enteric bacteria. It will be interesting to discover whether the mechanisms involving interaction at –100 to –400 differ from those of the more common positive regulatory sites at –50 to –90, which are thought to involve either direct interaction with RNA polymerase or local changes in DNA structure.

Attempts to exploit these global regulatory circuits to enhance transcription have involved overproduction of the positive regulatory factors either by increasing their copy number or by using hyperproducing mutants such as *sacU^h*. A *sacU^h* strain showed a 5- to 10-fold increase in expression of a *xylE* reporter gene from the *apr* (subtilisin) promoter, and a 50- to 100-fold increase from the *sacB* (levansucrase) promoter (Zukowski and Miller, 1986). In these experiments the *xylE* gene was carried on a multicopy plasmid and expression was largely confined to stationary phase. The *sacU^h*, *paq*, and *amyB* mutations are all thought to lie in the same gene (Steinmetz *et al.*, 1976) and are probably promoter-up mutations. Overproduction of the *B. subtilis* *sacQ* product has a less dramatic effect on expression from these promoters than does the *sacU* product. Thus levels of levansucrase and subtilisin are lower in both a *sacQ^h* strain and a strain carrying the cloned *B. subtilis* *sacQ* gene than in a *sacU^h* strain (Amory *et al.*, 1987). Higher level were, however, conferred by the presence in high copy of the *B. licheniformis* *sacQ* gene, or a modified version of the *B. amyloliquefaciens* *sacQ* gene (Amory *et al.*, 1987; Sloma *et al.*, 1987). Integration

and amplification in the *B. subtilis* chromosome of the *B. licheniformis* α -amylase gene expressing from the *B. subtilis* *apr* promoter allowed secretion and accumulation of the amylase to about 50 mg/liter. Introduction into this strain of the modified *B. licheniformis* *sacQ* gene on a multicopy plasmid and expression from a strong promoter led to a 25-fold increase in this level, i.e., to about 1 g/liter (Sloma *et al.*, 1987). One limitation of this approach is that overproduction of a pleiotropic activator can lead to an increase in protease production, with consequent problems for the stability of heterologous proteins.

The second general strategy involves the use of combinations of well-characterized promoters, operators, and repressors. In the first example of this approach (Yansura and Henner, 1984b), control of transcription from a phage SP01 promoter was achieved using the *E. coli* *lac* repressor and operator. The expression plasmid carries the *lac* repressor gene expressed from the weak constitutive *B. licheniformis* *penP* promoter and ribosome-binding site, and the target gene preceded by the SP01 promoter and *lac* operator. Transcription of foreign genes from this "*spac*" promoter shows 50- to 100-fold induction in response to IPTG. This controllable system permitted the maintenance of a particularly deleterious *B. licheniformis* penicillinase gene on a high copy number plasmid in *B. subtilis*. It also permitted controlled expression of leukocyte interferon and human growth hormone, the latter with intracellular accumulation to about 1.5 g/liter (Ruppen *et al.*, 1985).

A similar system has been developed which uses the *E. coli* *lac* regulatory elements to control the powerful phage T5PN26 promoter (Le Grice *et al.*, 1987). This system, in which the *lac* repressor is supplied *in trans* from a second, compatible plasmid, has been used for controlled expression of HTLV genes in *B. subtilis*. Both these promoter systems appear to be efficient in that they permit tight control and high induced levels of expression. Induction, however, requires the use of IPTG, which is clearly not economically feasible for producing low-value products on a large scale. The isolation of temperature-sensitive *lac* repressor mutants might enable the development of more economically attractive thermoinducible variants of these systems.

The need for tight regulation and relatively cheap induction has generated interest in the regulatory elements of sucrose catabolism in *B. subtilis*. Regulation of *sacB*, the structural gene for levansucrase, is complex, involving several different positively acting components. The *sacB* promoter is constitutive but, in the absence of sucrose transcription of the *sacB* coding region, is prevented by an upstream terminator. In the presence of sucrose a positive regulator encoded by *sacS* prevents stem loop formation in the terminator (Aymerich and Steinmetz, 1987) and the gene is transcribed. The constitutive *sacB* promoter is moderately strong, giving expression of a *xylE* reporter gene on a multicopy plasmid to about 0.5% of total cell protein. In a *sacU^h* strain, however, expression from this promoter is increased 50- to 100-fold in the absence of the upstream terminator, allowing the *xylE* product to accumulate to about 25% of total cell protein (Zukowski and Miller, 1986). Most of this expression takes place in stationary phase, even though expression of *sacB* is normally confined to the growth phase. Reintroduction of the upstream terminator in this system was found to permit tight control over expression but induced levels in response to sucrose were much lower, possibly due to shortage of the *sacS* product. Cloning the *sacS* positive regulator onto the expression plasmid elevated the induced level somewhat. Replacing the *sacB* promoter with the powerful

T5PN26 promoter elevated it further but still permitted tight control over expression in the absence of sucrose. Induction in response to sucrose in this promising promoter system is rather slow and requires very high levels of sucrose (Zukowski *et al.*, 1987).

Although a number of very powerful inducible promoters have now been developed, their usefulness for expressing genes with secreted products still remains to be determined. Ideally, such promoters would be used to permit stable maintenance through the growth phase, followed by a burst of synthesis and secretion of the target gene product over a limited time in order to shorten fermentation times and minimize exposure to proteases. It appears likely that, for some secreted products at least, high-level expression following induction may saturate the secretion machinery of the cell, with very deleterious consequences (Nugent and Mountain, unpublished). More flexible promoters will need to be developed which, in addition to tight control and cheap induction, permit a range of induced activities such that the system can operate slightly below the maximum secretion rate for the particular target protein being expressed (see Section 8).

6.5. Transcription Terminators

Much less attention has been paid to transcription termination signals in *B. subtilis* than to promoters. *Escherichia coli* terminators fall into two classes, which are respectively dependent on and independent of the presence of a protein factor ρ . The attenuators involved in regulation of some amino acid biosynthetic operons may be regarded as somewhat more complex and modulated versions of the ρ -independent type. ρ -dependent terminators of *E. coli* so far appear to show no striking sequence or structural homologies, whereas ρ -independent terminators characteristically show a GC-rich region of dyad symmetry in the template DNA followed by a series of T residues in which transcription is terminated. It is believed that the crucial termination signal to RNA polymerase resides in the mRNA rather than the DNA template: the dyad symmetry permits the formation of intramolecular hairpin structures in the mRNA which cause the polymerase to pause, while the uridines facilitate dissociation of the transcript from the template (see Platt, 1986). Very few *Bacillus* terminators have been studied in detail, although sequences strikingly similar to ρ -independent terminators have been observed at the 3' end of many genes. The exact sites of transcription termination in *B. subtilis* have been determined for the following genes: the *spoOA* locus (Ferrari, F. A., *et al.*, 1985a), *gnt* operon (Fujita *et al.*, 1986) of *B. subtilis*; the *cat86* gene of *B. pumilus* (Mongkolsuk *et al.*, 1985); the α -amylase gene of *B. amyloliquefaciens* (Lehtovaara *et al.*, 1984); the *cry* gene of a strain of *B. thuringiensis* (Wong and Chang, 1986); and a $\phi 29$ gene (Pulido *et al.*, 1987). All six of these terminators have a relatively stable GC-rich symmetrical sequence but only in the case of the first three bacterial genes and the $\phi 29$ phage gene does the transcript terminate at a run of T residues. The modulated terminator in the *B. subtilis* *trp* attenuator has a GC-rich symmetrical sequence and the leader mRNA terminates, in the presence of excess tryptophan, at a run of T residues (Shimotsu *et al.*, 1986).

It appears that *Bacillus* terminators are similar to those of *E. coli* in both sequence and structural features. It is therefore not surprising that a number of ρ -independent *E. coli* terminators have been found to function efficiently in *B. subtilis*

(Peschke *et al.*, 1985). The λt_0 and *rrnB* T2 terminators are utilized extremely efficiently in *B. subtilis*, the former more efficiently than in *E. coli*. These well-characterized heterologous terminators are certain to prove valuable in the development of expression plasmids for *B. subtilis*, since they will reduce plasmid destabilization resulting from transcription readthrough. However, this may not be the only advantage of using strong termination signals: the highly efficient terminator from the *cry* gene of a *B. thuringiensis* strain has been shown capable of enhancing expression of several different genes in both *E. coli* and *B. subtilis* by mRNA stabilization (Wong and Chang, 1986). This terminator forms an extremely stable stem loop structure which increases very significantly the half-lives of mRNAs. Two other terminators from very long-lived bacterial mRNAs, those of the *E. coli lpp* and *S. aureus* pE194 *erm* genes, appear not to have similar effects (Wong and Chang, 1986).

6.6. Transcription Regulatory Circuits

Among the regulatory circuits which are being studied at the molecular level in *B. subtilis*, and which may eventually provide components for controllable expression systems, are those of tryptophan, leucine, and arginine biosynthesis; sucrose, xylose, and gluconate utilization; and lysogeny in phage $\phi 105$. Analysis of the regulation of penicillinase synthesis in *B. licheniformis* is also quite advanced. There is good evidence that expression of the *B. subtilis trp* operon is regulated largely by a novel form of transcription attenuation (Shimotsu *et al.*, 1986), which involves alternative secondary structures in the leader mRNA but not ribosomal stalling. It is suggested that a tryptophan-activated regulatory protein binds to a specific segment of the nascent leader transcript, preventing formation of one of the alternative secondary structures and thereby directing RNA polymerase to terminate transcription (Kuroda *et al.*, 1988). Similar alternative secondary structures are predicted on the basis of DNA sequence information to be able to form in the leader mRNA of the leucine operon (Zahler, personal communication). For the major *arg* operon, on the other hand, there is no evidence for regulation by transcription termination and repression in the presence of arginine appears to be exerted largely at the level of transcriptional initiation at the *arg* promoter (Smith *et al.*, 1986). The regulatory gene for this system, *ahrC*, has been sequenced and the predicted product shows homology to that of the *E. coli argR* gene. Significant homology has also been observed between the *E. coli arg* operators and three imperfect inverted repeats located between -64 and +9 in the *B. subtilis arg* promoter which are suggested to be involved in the repression mechanism (Smith *et al.*, 1986). In the arginine system the structure and function of the regulatory and structural genes of *B. subtilis* and *E. coli* are highly conserved despite fundamental differences in habitats and life-styles.

Control of expression of the levansucrase gene (*sacB*) has been described in simplified form above: induction in the presence of sucrose involves positive regulation of premature transcription termination (Aymerich and Steinmetz, 1987). Transcription from the *sacB* promoter is constitutive but is stimulated by a whole series of positively acting global regulatory factors. Expression of the five structural genes encoding the enzymes of xylose utilization appears to be subject to negative

control by a repressor, which is the product of a gene *xylR* located within the same gene cluster (Hastrup, 1987; Gartner *et al.*, 1988). Xylose induces the operon by inactivating the repressor. *xylR* has been cloned and its product is believed to interact with symmetrical operator sequences overlapping the *xyl* promoters. Expression of the *gnt* operon, encoding the enzymes of gluconate utilization, appears to be regulated in a similar fashion except that the *gntR* repressor gene is located within the same transcription unit as the structural genes (Fujita and Fujita, 1986; Fujita *et al.*, 1986; Fujita and Farnie, 1987; Miwa and Fujita, 1987).

Although it is very likely that the *arg*, *gnt*, and *xyl* operons are subject to negative control by repression, the only regulatory system in *B. subtilis* for which there is definitive proof so far of classical repressor-operator interactions is that which maintains lysogeny in the temperate phage $\phi 105$. The product of the phage repressor gene (*c*₁₀₅) tightly controls transcription from the adjacent phage early promoter by interaction with a 14-base pair operator sequence. Mutations rendering expression of a reporter gene from the promoter constitutive are found to be located either within the repressor gene or in the operator (Van Kaer *et al.*, 1987). In fact, the general strategy employed by $\phi 105$ to control lysogeny appears at this stage of analysis to be similar to that used by the lambdoid phages of *E. coli*, in that the *c*₁₀₅ product also acts as a positive regulator by stimulating transcription from its own promoter. For $\phi 105$, however, the operator at which the repressor exerts its negative regulation is located 250 base pairs downstream of the early promoter. This 14-base pair operator is unique among all operators so far described in prokaryotes in that it completely lacks twofold rotational symmetry, suggesting that repressor binding may not be cooperative. The repressor exerts the kind of tight control over expression from the early promoter that is required for stable maintenance of an expression plasmid carrying a target gene. Two groups have used these regulatory elements to develop a two-plasmid control circuit which comprises the promoter and operator fused to a reporter gene on a pUB110 derivative and, present in the same cell, the repressor gene carried on a compatible plasmid, pE194 (Dhaese *et al.*, 1984; Osburne *et al.*, 1985). Because replication of the latter is temperature-sensitive in *B. subtilis*, growth at the nonpermissive temperature permits induction of expression from the promoter through gradual loss of the repressor plasmid. Although the early phage promoter is too weak, even when fully induced, to give high expression levels, it has proved possible to use the repressor/operator combination to control expression from somewhat stronger promoters (Van Kaer *et al.*, 1987). It is probable that an expression plasmid could be developed which would use the operator and a temperature-sensitive repressor to achieve thermoinducible expression from a strong promoter.

Expression of the β -lactamase gene (*penB*) of *B. licheniformis* also appears to be under negative control by repression (Himeno *et al.*, 1986). The repressor is believed to regulate expression of β -lactamase, itself and an "antirepressor" by interacting with symmetrical 23-base pair operator sequences (Himeno *et al.*, 1986; Imanaka *et al.*, 1987; Kobayashi *et al.*, 1987; Grossman and Lampen, 1987). Induction of β -lactamase by β -lactam antibiotics is thought to involve cooperative functions of the inducer, repressor, and antirepressor (Imanaka *et al.*, 1987). The antirepressor may be a membrane receptor for β -lactams and is homologous to a penicillin-binding protein of *E. coli*. A number of expression/secretion plasmids

have been constructed using the inducible *penB* promoter and signal sequence (Chang *et al.*, 1982; Himeno *et al.*, 1986) which are suitable for laboratory scale production.

It can be seen from the above brief summary that strains of *Bacillus* employ many of the familiar mechanisms used by *E. coli* and its close relatives as well as some encouragingly novel ones of its own.

7. TRANSLATION

Among the factors found to influence translation efficiency in *E. coli* and which might, by analogy, limit expression levels for cloned genes in *B. subtilis* are mRNA stability, rate of translation initiation, and codon usage. Little is yet known about the factors affecting mRNA stability in *B. subtilis* beyond the report of stabilization by an extremely efficient transcription terminator mentioned in Section 6.5. There is, however, considerable evidence illustrating the importance of the ribosome-binding region on the mRNA in determining the level of expression of cloned genes in *B. subtilis*. It is well known that although *E. coli* is capable of recognizing and utilizing most vegetative promoters and ribosome-binding sites in cloned genes from gram-positive organisms, including those of *B. subtilis*, the majority of cloned gram-negative genes examined fail to express from their own expression signals in gram-positive hosts (with the notable exception of Streptomyces). It now appears that expression of *E. coli* genes in *B. subtilis* generally fails at the level of initiation of both transcription and translation.

Examination of ribosome-binding regions in *E. coli* suggests that the following features are important in determining the frequency of translation initiation: a region of complementarity between the 3' end of 16S ribosomal RNA and a sequence 5–15 bases 5' to the initiation codon which has become known as the "Shine–Dalgarno" (SD) sequence; the spacing between the SD sequence and the initiation codon; the initiation codon itself; and the mRNA secondary structure in this region. In *E. coli* the region of complementarity is usually comparatively short and typically involves the sequence AGGA on the mRNA, with a spacing of 6–12 bases to the initiation codon, which is almost always AUG or GUG (Gold *et al.*, 1981). It was realized some years ago (McLaughlin *et al.*, 1981) that gram-positive mRNAs generally have a more extensive region of complementarity, with free energies of interaction between the SD region and the 3' end of 16S ribosomal RNA averaging around -17 to -18 kcal/mole for gram-positive bacteria and around -11 kcal/mole for *E. coli*. Table II shows the sequences of the ribosome-binding regions for seven *B. subtilis* genes which have intracellular products and are expressed at high levels, together with those of five with products which are secreted and accumulate in large quantities but which are probably expressed at a somewhat lower rate and for a longer period. Over a 12-base pair region close to the initiation codon the smallest number of bases complementary to the 3' end of 16S ribosomal RNA for all these genes is six, and the very highly expressed ones have at least seven with a minimum of six consecutive. It should be noted, however, that the use of sufficiently powerful promoters can permit high-level expression of some genes with only five complementary bases. The pC194 *cat* gene, for example, which features as the reporter gene in the multicopy promoter-probe plasmid pCPP4

Table II. Translation Initiation Regions of Genes Showing Efficient Expression in *Bacillus subtilis*

		Complementary			Spacing from	References
		nucleotides			GAGG to ATG	
16S ribRNA 3' end		U U U C C U C C A C U A				
ideal" RBS		A A A G G A G G T G A T				
		AT-rich (spacer)				
Genes with abundant intracellular products	<i>rpmH</i>	A A g G G A G G T G t c a t a a A T G	12	8 or 9		Moriya <i>et al.</i> , 1985
	<i>rpmA</i>	A t A G G A G G T G A g c t a c A T G	8	8		F.A. Ferrari <i>et al.</i> , 1985
	<i>rpsK</i>	A A g G G A G G T t c T a a a c a A T G	10	8		Suh <i>et al.</i> , 1986
	<i>sspA</i>	c A A G G A G G T G A g a c a c A T G	9	9		Connors <i>et al.</i> , 1986
	<i>sspB</i>	A A A G G A G G a T t t T a c a c A T G	10	8		Connors <i>et al.</i> , 1986
	<i>rpoA</i>	g A A G G A G G g t t T t a g t a A T G	9	8		Suh <i>et al.</i> , 1986
Genes with secreted products	<i>rpoD</i>	g A A G G A G G a t c c a t a A T G	8	9		Wang and Doi, 1986
	<i>sacB</i>	A A A G G A G a c a t g a a c g A T G	7	8		Steinmetz <i>et al.</i> , 1985
	<i>bgl</i>	A A A G G g G a a t g c c a a t a A T G	6	9		Murphy <i>et al.</i> , 1984
	<i>aprA</i>	A A A G G A G a g G g T a a a g a G T G	9	9		Stahl and Ferrari, 1984
	<i>nprE</i>	A A A G G g G a t t T a t t G T G	8	7		Yang <i>et al.</i> , 1984
	<i>amyE</i>	A t A a G g a G T G t c a a g a A T G	6	8		Yang <i>et al.</i> , 1983
Reporter genes in common use	pC194 <i>cat</i>	t t c G G A G G c a t a t c a a A T G	5	8		Band <i>et al.</i> , 1983
	<i>xyIE</i>	t g A a G A G G T G A c g t c A T G	8	7		Zukowski <i>et al.</i> , 1983
	<i>cat-86</i>	t c A G G A G G a G A T a a a T T G	9	8		Duvall <i>et al.</i> , 1983

(Band *et al.*, 1983), can give chloramphenicol acetyltransferase (CAT) to at least 10% of total cell protein (Nugent and Mountain, unpublished). It seems likely though that ribosome-binding sites with only five complementary bases only function efficiently in *B. subtilis* if these are the highly conserved and consecutive GGAGG. It is possible that some genes, with ribosomal-binding sites containing less than five consecutive complementary bases, may achieve fairly efficient translation initiation through possession of multiple weak ribosome-binding sites, as suggested for the *B. subtilis* amylase gene (Yang *et al.*, 1983). The spacing between the final G of the GGAGG and the initiation codon varies between seven and nine bases for all 12 *B. subtilis* genes in Table II, and for the seven very highly expressed ones is either eight or nine bases.

An expression plasmid carrying one of the strong, directly controllable promoters described in Section 6.6 has been used to test the idea (McLaughlin *et al.*, 1981) that *B. subtilis* requires the more extensive region of complementarity in order to form a productive translation initiation complex, and to identify other features which may be important in determining the efficiency of translation (Band and Henner, 1984). Expression of a cloned leukocyte interferon gene in both *B. subtilis* and *E. coli* was measured using a series of plasmids which differed only in the translation initiation region of the gene. Expression was found to increase in *B. subtilis* but decrease in *E. coli* as the number of complementary base pairs increased from 4 to 7, and from 7 to 12. Four complementary bases gave virtually no detectable expression in *B. subtilis* but good expression in *E. coli*. These experiments also indicated the importance of the base composition in the spacer region, A's and T's giving much higher expression than G's and C's in *B. subtilis*, with this effect much less marked in *E. coli*. The length of the spacer appeared to be less important, consistent with the results of several studies in *E. coli* which suggest that the optimal spacing for very high-level expression has to be determined empirically. Plasmids permitting extremely high-level expression in *E. coli* therefore often have restriction sites located in the spacer region to permit the length of the spacer to be conveniently varied.

The effects of secondary structure at translation initiation sites in prokaryotes have recently been reviewed (Ganoza *et al.*, 1987). There are many instances where stem loop structures can be postulated with the initiation codon buried in the loop or exposed in the stem, and it has been postulated that loop-exposed AUG or GUG correlates with efficient expression (Iserentant and Fiers, 1980). Comparison of potential secondary structure around translation start codons with those surrounding internal methionines for a large number of prokaryotic genes revealed in general that start codons tend to lie in regions with little local pairing potential, and has led to the suggestion that the transition from relatively unstructured start domains to highly structured internal sequences may be an important determinant of translational start site recognition (Ganoza *et al.*, 1987). It is likely that very high level gene expression in *B. subtilis* will require the absence of secondary structures in which the translation initiation region is stably base-paired. The importance of such secondary structures is exemplified by the *cat-86* gene of *B. pumilus* and the *ermC* gene originating from *S. aureus* plasmids. For these genes induction in *B. subtilis* by subinhibitory levels of chloramphenicol or erythromycin, respectively, is believed to destabilize the stem loop structures which otherwise sequester the ribosomal binding sites (Duvall *et al.*, 1983; Bechhofer and Dubnau, 1987). Destabilization by

erythromycin has been shown to involve ribosomal stalling in the *ermC* leader peptide region, which also apparently confers an increase in the half-life of the *ermC* mRNA from 2 to 40 min (Bechhofer and Dubnau, 1987).

The translation initiation codon in *B. subtilis*, as for *E. coli*, can be AUG, GUG, or UUG. It is not clear whether the particular initiation codon used influences the level of gene expression, but the relative frequencies with which they occur in the *Bacillus* genes so far sequenced is the same as that in *E. coli*, AUG > GUG > UUG. It may be significant that for all seven of the highly expressed *B. subtilis* genes in Table II the initiation codon is AUG. So far the only UUG initiation codon among sequenced *B. subtilis* genes occurs in the *argC* gene (Smith *et al.*, 1986), which is relatively poorly expressed. Translation of the *cat86* gene from *B. pumilus*, however, is believed to initiate with a UUG (Harwood *et al.*, 1983), and this gene can be expressed to a high level in *B. subtilis* using strong promoters cloned into the promoter-probe plasmid pPL603. In *E. coli* replacement of the UUG initiation codon of the adenylate cyclase gene with AUG or GUG results in an increase in expression.

Sequence analysis has revealed that many genes from both *E. coli* and *Saccharomyces cerevisiae* have a marked codon bias, i.e., a nonrandom usage of alternative synonymous codons. In both species this bias is most obvious in highly expressed genes, which have high frequencies of codons thought to be optimally translated by the most abundant tRNA species. A recent analysis of codon usage data for 56 *B. subtilis* genes concluded that this organism in general displays much less codon bias than either *E. coli* or *S. cerevisiae* (Shields and Sharp, 1987). Nevertheless a set of genes was identified with relatively high codon bias, which in fact comprise the first five genes in Table I. These are the very highly expressed genes encoding ribosomal proteins and small, acid-soluble spore proteins. Although the relative abundances of the various tRNA species in *B. subtilis* are unknown, in most cases where a tRNA species is known to be encoded by more than one gene (Shields and Sharp, 1987) the codon translated (without wobble) is a preferred codon in this highly expressed group of genes. Certain codons were observed to be strongly avoided in this group of genes, and it is possible that the presence of a cluster of such codons in the target gene of a *B. subtilis* expression system could limit product yield, as has been observed for *E. coli* (Robinson *et al.*, 1984).

8. SECRETION

8.1. Secretion of Homologous Proteins

There are several recent reviews covering the secretion of proteins from bacteria in general (Holland *et al.*, 1986), from *Bacillus* (Sarvas, 1986), and from *E. coli* (Oliver, 1985), and of foreign proteins from microorganisms in general (Nicaud *et al.*, 1986). A further relevant review covers protein "topogenic" sequences involved in the movement of proteins through membranes (Robinson and Austen, 1987). The secretion of enzymes of industrial significance by *Bacillus* species is also discussed in Chapter 11.

Most species of *Bacillus* have a capacity to secrete a variety of proteins which

greatly exceeds that of gram-negative bacteria. Probably this partly reflects the relatively simple structure of the cell envelope, so that exported proteins need only traverse one membrane—the cytoplasmic membrane—to be exposed and released from the cell. There are basically two types of exported *Bacillus* proteins, the first being the “true” soluble exoproteins, mainly exoenzymes, which are often secreted in large quantities (several grams per liter) directly into the growth medium with very little, if any, remaining cell-associated. Typical examples are extracellular degradative enzymes such as amylases and proteases. The second category comprises a heterogeneous group of proteins which to some extent remain associated with the cell wall and/or cytoplasmic membrane, including autolytic enzymes, peptidoglycan-associated proteins such as staphylococcal protein A, and lipoproteins such as *B. licheniformis* β -lactamase.

Conceptually, secretion may be divided into two components: the machinery required and the information residing in the protein which is recognized by that machinery as an export signal. The great majority of proteins which are secreted from both bacteria and eukaryotes have in common a region at the N terminus, the signal peptide, which is, in part at least, responsible for directing the protein into the secretion pathway and which is cleaved by a signal peptidase during translocation through the membrane. Although signal peptides show little homology in primary amino acid sequence, they do show common structural features as depicted in Fig. 2. There is invariably an N-terminal hydrophilic and basic sequence followed by a stretch of uncharged residues, the “hydrophobic core.” The signal peptides of *Bacillus* exoproteins tend to be longer than those in gram-negative organisms, averaging 30–40 and 22 amino acids, respectively. Since the few Staphylococcal and Streptomyces signal peptides so far analyzed are also 30–40 residues, it may be that relatively long signal peptides are a general feature for gram-positive organisms. In *Bacillus* signal peptides there is also a tendency toward longer noncharged regions (averaging 14–23 residues) and more basic hydrophilic regions (with an average of four charged residues). It is not clear whether these unusual features have any functional importance. It may be relevant that in general *Bacillus* signal peptides appear to be compatible with efficient translocation of their exoenzymes across the inner membrane and into the periplasm of *E. coli*, whereas the periplasmic *E. coli* enzyme β -lactamase, for example, is not apparently secreted from *B. subtilis* using its own signal peptide (H. Smith, personal communication). The amino acid sequence at the cleavage or processing site of *Bacillus* signal peptides appears to conform to the general pattern for prokaryotes. Thus the available data indicate that cleavage generally occurs after an alanine residue or, in the case of lipoproteins, after a glycine residue (see Sarvas, 1986). There is often ambiguity, however, in determining the exact cleavage site because the N termini of the mature proteins are very commonly “nibbled” by secreted proteases, and also in some cases because further specific processing occurs following the removal of the signal peptide (see Section 8.2).

The secretion process in *Bacillus* is less well characterized than in *E. coli* and mammalian cells. Experiments have indicated that at least some *Bacillus* exoproteins are synthesized by membrane-bound ribosomes and are translocated across the membrane before their synthesis is complete (Sarvas, 1986, and references therein). There is circumstantial evidence that several proteins found in membrane-ribosome complexes in *B. subtilis* are components of the secretion machinery (Mar-

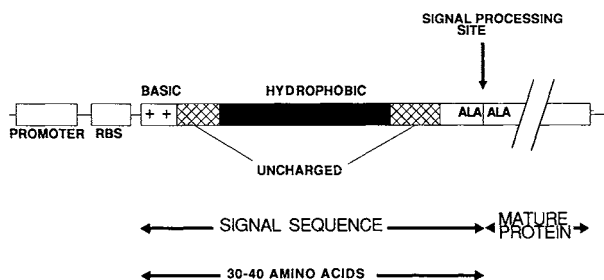


Figure 2. Structure of signal sequences in *B. subtilis*.

ty-Mazars *et al.*, 1983; Horiouchi *et al.*, 1983a,b; Caulfield *et al.*, 1984). A 64-kDa protein is particularly implicated, and a 46-kDa membrane protein, which may be the product of the *sacU* gene, may also be involved (Aubert *et al.*, 1985). Although no signal peptidases have yet been characterized for *B. subtilis*, it is likely that this organism will prove to resemble *E. coli* in possessing two such enzymes, one for cleaving the signal peptide from lipoproteins and the other for cleavage from other secreted proteins.

8.2. Secretion of Heterologous Proteins

The available evidence indicates that gram-positive exoproteins in general can be secreted efficiently from *B. subtilis* using their own signal sequences. Thus all amylases, proteases, nucleases, cyclodextrin glucosyltransferases, and β -lactamases cloned from other species of *Bacillus* and expressed in *B. subtilis* appear to be efficiently exported into the medium. Similarly, the signal sequences from a β -lactamase (Wang, P-Z., *et al.*, 1987), a nuclease (Kovacevik *et al.*, 1985), and Staphylococcal protein A (Fahnestock and Fisher, 1986) from *S. aureus* all give efficient export of their respective mature proteins, as does that of a carboxymethylcellulase from *Clostridium thermocellum* (Soutschek-Bauer and Staudenbauer, 1987). For such proteins, then, it is probably not necessary to replace the signal peptide with one from *B. subtilis* in order to achieve secretion, although it may often be technically more convenient to do so.

No detailed studies have been reported on the ability of the shorter signal peptides from gram-negative organisms to direct secretion from *B. subtilis*, but that of *E. coli* TEM β -lactamase is apparently unable to do so (H. Smith, personal communication).

Attempts to use the secretion signals of *Bacillus* exoproteins to secrete heterologous products have mainly concentrated on those of the true exoenzymes which are secreted in large quantities, particularly those of amylases and proteases. All the *Bacillus* protease genes so far sequenced contain a large open reading frame (100–220 amino acids) between the regions coding for signal sequence and mature protein, so that the primary translation product has a pre-pro-mature structure reminiscent of eukaryotic secreted proteases. A full-length precursor of transient existence and corresponding in size to the primary translation product has been observed inside the cell in pulse-labeling experiments for both the alkaline and

neutral proteases of *B. amyloliquefaciens* (Vasantha *et al.*, 1984), demonstrating post-translational translocation for these proteins. The function of the propeptide is unknown, but it is probably not involved in translocation. It may instead render the protease inactive when close to protease-sensitive components of the cell membrane or cell wall, the protease being activated later by an autocatalytic process. It has also been suggested that its net positive charge might serve to bind the protein to the cell membrane or cell wall (Vasantha *et al.*, 1984). The propeptides are probably not unique to *Bacillus* proteases: some other exoproteins may have similar although smaller regions between the signal sequence and mature protein, e.g., a staphylococcal nuclease (Miller *et al.*, 1987) and the α -amylase (Yang *et al.*, 1983) and β -glucanase (Murphy *et al.*, 1984) of *B. subtilis*. The β -lactamase of *B. licheniformis* is a lipoprotein synthesized as a precursor from which the signal peptide is cleaved during translocation to give a hydrophobic, membrane-bound precursor. Two further cleavages close to the N terminus can then lead to release of relatively hydrophilic extracellular forms (Chang *et al.*, 1982).

Secretion vectors have been developed using promoters, ribosome-binding sites, and secretion signals from the α -amylase genes of *B. subtilis* (e.g., Shiroza *et al.*, 1985; Yamane *et al.*, 1986) and *B. amyloliquefaciens* (Palva, 1982; Ulmanen *et al.*, 1985; Fahnestock and Fisher, 1986), the alkaline protease gene of *B. subtilis* (Wong *et al.*, 1986), both the alkaline and neutral protease genes of *B. amyloliquefaciens* (Vasantha and Thompson, 1986), the β -lactamase gene of *B. licheniformis* (Chang *et al.*, 1982) and many others. Efficient expression/secretion has been achieved for a number of heterologous bacterial exoproteins using these secretion vectors by fusing the coding region of the mature proteins in frame and close to the end of the various signal sequences. Thus it has been shown that the periplasmic *E. coli* proteins TEM β -lactamase (Ulmanen *et al.*, 1985) and alkaline phosphatase (Stephens *et al.*, 1986) can be efficiently exported from *B. subtilis*, as can the S1 subunit of *Bordetella pertussis* toxin (Sarvas, 1986) and Staphylococcal protein A (Fahnestock *et al.*, 1986; Vasantha and Thompson, 1986). Efficient secretion of streptococcal protein G can also be achieved but only in constructs lacking the C-terminal membrane anchor region (Fahnestock and Fisher, 1987).

Data on the effect on secretion efficiency of the amino acid sequence surrounding the fusion junction between signal sequence and mature protein are somewhat conflicting: in some cases the yield of secreted product is severely affected by changes in this region while in others a variety of linker-encoded amino acids appear to be compatible with efficient export (Sarvas, 1986; Doi *et al.*, 1986). The highest yields of secreted heterologous proteins reported have been obtained using the signal sequences of the α -amylase and protease genes from *B. amyloliquefaciens* (Ulmanen *et al.*, 1985; Fahnestock and Fisher, 1986; Vasantha and Thompson, 1986). This does not necessarily imply that these signal sequences give faster or more efficient secretion than others, only that these combinations of promoter, ribosomal binding site, signal sequence, and signal sequence/mature protein gene junctions are particularly compatible with each other and with efficient export. There is no clear evidence that signal sequences from different sources direct secretion from *B. subtilis* with different efficiencies. Estimating the yield of heterologous products is complicated by protease degradation problems, but secretion and accumulation of Staphylococcal protein A up to 3 g/liter has been achieved in protease-defective strains (Fahnestock and Fisher, 1986; Vasantha and Thompson, 1986). This yield is very similar to that of the natural amylase or protease from

which the expression and secretion signals were derived. The highest yields of heterologous proteins are often achieved with constructs in which the mature protein is fused at least one amino acid 3' to the end of the signal sequence, such that the normal processing site is left intact. Where the absence of protease "nibbling" has permitted N-terminal sequence analysis of the secreted products, the results suggest that the signal peptide is cleaved in the normal position (Sibakov *et al.*, 1984; Vasantha and Thompson, 1986; Doi *et al.*, 1986). This leaves one or more extra amino acids on the N terminus of the mature protein, which may be disadvantageous from the viewpoint of acceptability to regulatory authorities, particularly when the product is for health care applications. There are examples, however, of accurate and efficient cleavage for precise fusions between the C-terminal amino acid of the signal peptide and the usual first amino acid of the mature heterologous protein (Sarvas, 1986; Vasantha and Filpula, 1987). There is evidence that even heterologous proteins which are apparently efficiently secreted and which can accumulate to high levels are secreted more slowly than the protein from which the signal sequence is derived. For example, *B. subtilis* cells carrying protease/Staphylococcal protein A fusions accumulate full-length precursor and at least 30% of the signal processing occurs after synthesis is complete, whereas the full-length precursor for the protease itself has only transitory existence and is very difficult to detect (Vasantha and Thompson, 1986). This may indicate that the maximum secretion rate varies for different proteins, and also perhaps for different constructs involving the same signal sequence and mature protein. There have been several observed cases that indicate that exceeding the secretion capacity of *B. subtilis* significantly has deleterious effects. For example, hybrid plasmids carrying the *spa* gene, encoding Staphylococcal protein A expressing from its own strong promoter, proved very difficult to establish and stabilize in *B. subtilis*, but replacement with the much weaker α -amylase promoter or insertion into the chromosome led to markedly increased stability (Fahnestock and Fisher, 1986). Similarly, expression of an α -amylase gene from a very powerful promoter on a multicopy plasmid has been found to exert very strong selection for deletion derivatives (Nugent and Mountain, unpublished). These results may profoundly affect the choice of promoters for expression/secretion systems in that powerful, directly controllable promoters may not be suitable for many secreted products.

Almost all secretion in *B. subtilis* takes place after the end of rapid growth for reasons that are not well understood. The only protein secreted in relatively large quantities during the growth phase is levansucrase, and even when fully induced by sucrose the enzyme does not accumulate to the very high levels required for commercial enzyme production. Although the *sacU^h* mutation dramatically increases expression and secretion of this enzyme, most of the expression then occurs in stationary phase (see Section 6). Almost all reports on secretion and accumulation of cloned gene products have used promoters, such as those of amylase and protease genes, which are weak and are relatively inactive until the onset of stationary phase. However, these promoters continue to be active for long periods in stationary phase. The translation initiation regions of such genes also typically appear to be only moderately efficient. The transcription and translation signals of such genes have presumably evolved this way for maximum compatibility with the secretion capacity of the organism. One recent report on secretion during the growth phase concerns the expression and secretion of TEM β -lactamase using the subtilisin signal sequence and an uncharacterized vegetative promoter (Wong *et al.*,

1986), but β -lactamase accumulation did not exceed 50 mg/liter. Thorough studies are needed to determine how maximum secretion rates vary with the protein secreted, with the growth phase, and with growth conditions such as temperature and media composition. The role of the cell wall in protein export also needs to be examined.

For *B. subtilis* there are no reports describing successful attempts to export proteins which normally have an intracellular location. Fusion of a CAT coding sequence (encoding chloramphenicol acetyltransferase) in frame with the *B. licheniformis* β -lactamase secretion and expression signals on a shuttle plasmid gave a hybrid which proved deleterious in *E. coli* and lethal in *B. subtilis* (Doi, 1984). There is also one specific class of exported proteins which *B. subtilis* is unable to secrete, namely, the outer membrane proteins of *E. coli*. These are inserted into and translocated across the cytoplasmic membrane of *E. coli* in a fashion very similar to the soluble periplasmic proteins, the gram-negative analogs of secretory proteins. Use of the same secretion vector which permitted efficient export from *B. subtilis* of *E. coli* β -lactamase and several other heterologous proteins allowed the synthesis of large amounts of both *ompA* and *ompF* products, but these remained in the cell with the signal peptide unprocessed (Kallio *et al.*, 1986; Sarvas, 1986).

There are very few reports of efficient secretion from *B. subtilis* and accumulation to relatively high levels of eukaryotic exoproteins. Such proteins appear to be extremely susceptible to degradation by secreted proteases, which makes it particularly difficult to distinguish between efficient secretion and rapid hydrolysis. It is only fairly recently that thorough experiments have been reported involving pulse-labeling procedures and protease-defective strains. Secretion and accumulation to low levels (a few milligrams per liter) have been achieved for human α -interferon (HAI; Schlein *et al.*, 1986), mouse β -interferon (MBI; Shiroza *et al.*, 1985), human β -interferon (HBI; Honjo *et al.*, 1985), human interleukin-1 (HIL; Motley and Graham, 1987), bovine prochymosin and pancreatic ribonuclease (BPR; Vasantha and Filpula, 1987), and human serum albumin (HSA; Saunders *et al.*, 1987). In the best studied cases—HAI, BPR, and HSA—the proteins were expressed and secreted using vectors known to permit high-level secretion and accumulation of heterologous bacterial exoproteins. In the case of HAI an amylase gene-based secretion vector was used that secreted HAI to only 1–3% of the level observed for the α -amylase itself. Messenger RNA levels for the two proteins were shown to be similar. The low yield could be partly attributed to protease degradation, but in early stationary phase before protease activity became significant the secretion rate for HAI was shown to be 13-fold lower than that for amylase and the cells accumulated an insoluble AMY-HAI precursor which was unprocessed even for constructs in which the natural amylase signal cleavage site was preserved. The BPR studies used a protease gene-based secretion vector and found that imprecise fusions, i.e., preserving the protease signal cleavage site, gave virtually no secretion or processing. A precise fusion between the 3' terminal amino acid of the signal sequence and the normal amino acid at the 5' end of mature BPR permitted secretion with accurate processing, but with yields 50-fold lower than achieved for Staphylococcal protein A. The secreted BPR was immunologically active, suggesting that formation of its four disulfide bonds occurred spontaneously. Secretion of HSA using both amylase and protease gene-based secretion vectors was extremely poor and unreplicable. Pulse-labeling studies with protoplasts indicated that the protein could be efficiently translocated across the membrane, and with intact cells that the signal

peptide could be cleaved but that subsequent transfer to the medium was very inefficient. Signal peptide removal was efficient in cells expressing HSA at low levels but not at high levels, possibly because the latter leads to accumulation of HSA in an aggregated insoluble form, either inside the cell or on the external surface of the membrane.

These results suggest that *B. subtilis* has very limited capacity to export most eukaryotic proteins, and this limitation may be due to their inefficient translocation, processing, or subsequent passage through the cell wall. Exceeding this capacity may, in many cases, lead to the aggregation of the protein in an insoluble form which is not compatible with secretion. Mutant strains with increased secretion capacity for such proteins are currently being sought, e.g., by screening for larger clearing zones surrounding colonies secreting BPR on plates containing RNA. It is not clear why the secretion capacity of *B. subtilis* is lower in general for eukaryotic exoproteins than those of bacteria, but it may be that the protein structural requirements (or requirements for lack of tertiary structure) for export are less stringent in at least some eukaryotic cells than in bacteria. There is at least one report of secretion from mammalian cells of a protein which usually has an intracellular location (Williams and Neuberger, 1986).

Human growth hormone (HGH) may be a rare example of a eukaryotic protein which can be secreted relatively efficiently by *B. subtilis*: a protease gene-based secretion vector enabled secretion and accumulation of HGH to about 200 mg/liter, with accurate processing (Honjo *et al.*, 1987). This is the most encouraging result so far reported for the secretion of a eukaryotic protein from *B. subtilis*.

An alternative approach which may be particularly applicable to small eukaryotic peptides involves fusion of the peptide to the C terminal end of the entire amylase or protease rather than to their signal peptides. A secretion vector has been constructed which permits fusion in this position to the α -amylase of *B. licheniformis* by a specific protease cleavage site (Stephens *et al.*, 1986). Use of this vector allowed secretion and accumulation of an amylase-atriopeptin III fusion in quantities similar to those observed for the intact amylase (g/liter) followed by recovery of the 24-residue eukaryotic peptide from the fusion at 50% efficiency.

In summary, results so far indicate that it will soon prove possible to secrete efficiently most bacterial exoproteins from *B. subtilis*. However, the secretion capacity of this organism for most eukaryotic proteins appears at present to be strictly limited. It is possible that other species of *Bacillus* will prove to have a greater secretion capacity than *B. subtilis*. One particular strain of *B. brevis*, for example, has been observed to secrete up to 12 g/liter of a homologous protein into the growth medium (Ohmizu *et al.*, 1983). This very high secretion capacity is related to the unusual property of shedding two cell wall proteins into the medium. Attempts are under way to develop a recombinant secretion system for this organism using expression and secretion signals from the genes encoding these wall proteins (Tsukagoshi *et al.*, 1985).

9. PROTEASE DEGRADATION

Although some exoenzymes from related *Bacillus* species are not degraded by *B. subtilis* proteases, most heterologous secreted proteins are susceptible. Degradation usually begins in early stationary phase, when protease production and ac-

cumulation are rapidly increasing. Most of the extracellular protease activity of *B. subtilis* is attributable to two proteases, a neutral metalloprotease sensitive to EDTA and encoded by the *nprE* gene, and an alkaline serine protease (subtilisin) sensitive to PMSF and encoded by the *aprA* gene (Kawamura and Doi, 1984). Mutants defective in both these major proteases have been constructed by several groups (Stahl and Ferrari, 1984; Kawamura and Doi, 1984; Fahnestock and Fisher, 1987). These strains have protease activities in early stationary phase of 2–10% compared with their parent, as measured by nonspecific protease assays, but grow and sporulate normally. Although the degradation rate of some heterologous exoproteins is reduced in these strains, they do not generally allow accumulation of foreign proteins to high levels. For example, *E. coli* β -lactamase secreted from such a strain using a protease gene-based vector stops accumulating and begins to be degraded after about 6 hr in stationary phase (Doi *et al.*, 1986). Accumulation of product to high levels using amylase and protease gene-based secretion vectors requires at least 24 hr in stationary phase. It is not clear how many minor proteases are produced by the *nprE*, *aprA* strains, but most of the residual protease activity seems to be of the serine protease type. One which may be involved is bacillopeptidase F (Roitsch and Hageman, 1983).

Residual protease activity can be very significantly reduced by introduction of pleiotropic *spoOA* mutations into the *nprE*, *aprA* double mutant (Fahnestock and Fisher, 1987). The resulting mutant accumulates no residual metalloprotease activity and less than 1% of normal serine protease activity, and permits accumulation of the normally susceptible Staphylococcal protein A to very high levels (g/liter). Use of this strain, however, does not apparently permit accumulation of most secreted eukaryotic proteins, for which several minor proteases may yet need to be inactivated. The structural gene for one uncharacterized minor protease has recently been cloned (Bruckner and Doi, 1987) and a mutant defective for this and the two major proteases constructed, but its value remains to be determined. Introduction of a cloned *sacQ* gene has also been found to confer a very significant reduction in protease levels (Honjo *et al.*, 1987) and permits the accumulation of secreted human growth hormone.

One major worry concerning the development and use of strains lacking extracellular protease activity is the possibility that they may be unable to reach the very high biomass required for good product yield in cheap industrial media. Another concern is that they may prove more prone to autolysis, leading to the release of intracellular proteases into the medium.

Levels of secreted proteases have been observed to vary dramatically with the composition of the growth medium. Unfortunately, media giving low protease activities may also give rather poor expression of cloned genes, particularly for protease gene-based secretion vectors (Vasantha and Thompson, 1986). The presence of glucose in high concentration suppresses residual protease production sufficiently to permit the accumulation of *E. coli* β -lactamase secreted by an *nprE*, *aprA* strain (Doi *et al.*, 1986). The β -lactamase activity was undiminished after 100 hr of cultivation.

Since the production of secreted proteases occurs primarily in stationary phase, it may be possible to use directly controllable promoters to induce a burst of synthesis and secretion during the transition from growth to stationary phase in order to minimize exposure to proteases. As discussed in Section 8, the use of this strategy

would be limited to products with high maximum secretion rates, since it requires secretion to occur over a much shorter period of time. Although there are very few reports concerning secretion in the growth phase, a combination of weak vegetative promoters and high glucose media permits low-level production of intact *E. coli* β -lactamase (Doi *et al.*, 1986; Wong *et al.*, 1986) and HIL (Motley and Graham, 1987). There are as yet no reports on the use of powerful, directly controllable promoters for the expression of secreted products.

A more radical alternative may be possible for proteins whose degradation cannot be avoided using a combination of protease-defective *B. subtilis* strains, appropriate media, and timing of expression. This would involve developing expression/secretion systems for alternative *Bacillus* species which produce much lower protease levels. A particular strain of *B. brevis*, for example, has been reported to produce no detectable intracellular or extracellular protease activity in some media (Mezes *et al.*, 1985). The secretion capacity of this strain is unknown and, considering our ignorance of *Bacillus* gene expression mechanisms and molecular biology outside that of *B. subtilis*, this would clearly be a long-term approach.

The structural gene *isp-1* encoding the major intracellular serine protease of *B. subtilis* has been cloned (Koide *et al.*, 1986) and defective mutants have been constructed which may give increased yields of some heterologous proteins produced intracellularly (Stephens, personal communication).

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Genetic Manipulation of *Bacillus subtilis*

6

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1. INTRODUCTION

Bacteria have long been the focus of research areas with very different goals. Bacteria provide model cell systems in which it is possible to explore basic mechanisms of genetics, biochemistry, and physiology. Bacteria are also of importance to industry for the production of antibiotics, chemicals, and enzymes. The role of many bacteria in causing disease has been the subject of extensive and fruitful studies for many years and will continue to be so in the future. Lastly, the ecological importance of bacteria has long been recognized and in recent years this field has seen a resurgence of interest, particularly in the area of nitrogen fixation.

Common to all these studies is the concept of genetic manipulation, which involves the application of a wide array of technologies to generate, select, and characterize changes in the genotype of chosen bacterial systems. Genetic manipulation in bacteria can include mutagenesis and selection schemes as well as manipulations resulting from the application of the traditional genetic exchange techniques of transformation, conjugation, and transduction. Among the many procedures that are currently used in altering the genotype of bacteria, the past 15 years has seen the emergence of recombinant DNA technology, transposon technology, and methods of DNA sequencing and site-directed mutagenesis as the dominant approaches. These tools provide direct and unambiguous means of achieving specific genetic alterations. The universal application of these technologies reflects both their usefulness and relative simplicity.

The focus of this chapter is the application of recombinant DNA technology in *Bacillus subtilis* and related species. Unlike the techniques of DNA sequencing and site-directed mutagenesis, the development of recombinant DNA and transposon technologies requires vectors and methods that are frequently useful only among members of one group of bacteria. The reader is encouraged to examine previous reviews of this topic (Lovett, 1981, 1984; Gryczan, 1982; Youngman *et al.*, 1985).

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The concept of recombinant DNA technology has its origins in classical studies performed in *Escherichia coli* during the 1960s and early 1970s. Analysis of plasmids in *E. coli* demonstrated that each was a covalently closed circular (CCC) duplex DNA molecule and these were generally maintained in cells in an extra-chromosomal state (Roth and Helinski, 1967). The unique physical properties of plasmids enabled their rapid isolation in a very pure state by use of the dye-buoyant gradient centrifugation method originally developed in the laboratory of Vinograd (Radloff *et al.*, 1967). The ability of investigators to recover plasmids quantitatively from cells revealed that different plasmids were maintained at characteristic copy numbers. Thus, the relatively large F factor is maintained at a ratio of one or two copies per chromosome whereas many other plasmids are maintained at significantly higher copy numbers. The idea that plasmid replication could be separated from chromosome replication was shown by the demonstration that replication of the ColEI plasmid occurred when host translation, and consequently chromosome initiation, was blocked (Clewell, 1972). Similarly, the isolation of plasmid mutants that cannot replicate at elevated temperature further emphasized that, although the replication of plasmids and chromosomes shares common cell constituents, the replication processes are also replicon-specific.

Plasmid transformation in *E. coli*, using the technique developed for λ transfection, allowed analysis of the biological activity of purified plasmid DNA (Cohen *et al.*, 1972). These studies showed, for example, that covalently closed plasmid DNA transformed far more efficiently than nicked circles or linear plasmid forms. Moreover, since plasmid transformation did not change the physical or genetic properties of the molecules, this methodology provided a convenient alternative technique to transfer plasmids in *E. coli*, in addition to transduction and conjugation.

Between 1970 and 1972 three essential observations established the basis for the evolution of recombinant DNA technology in *E. coli* and subsequently in other bacteria. First, a known DNA restriction (and modification) system in *Hemophilus influenza* was correlated with the presence of an endonuclease that recognized a specific hexanucleotide sequence in DNA and made a double-strand scission at that site (Smith and Wilcox, 1970; Kelly and Smith, 1970). This enzyme, subsequently designated *Hind*II, proved to be the first of many sequence-specific endonucleases for DNA identified in various microorganisms. Second, the concept of joining together an *E. coli* replicon, λ , and an unrelated DNA molecule by "tailing" DNA fragments with complementary nucleotides using terminal transferase suggested a general method that would permit the maintenance of heterologous DNA in *E. coli* (Jackson *et al.*, 1972). Third, a site-specific endonuclease, *Eco*RI, was shown to produce self-complementary cohesive termini when it cleaved DNA (Metz and Davis, 1972). Thus, unrelated DNA molecules cut with this enzyme could be directly joined by annealing of the ends and the molecules then covalently sealed by DNA ligase. The demonstration of *in vitro* ligation of two unrelated plasmid molecules, each cut with *Eco*RI, and the successful transformation of *E. coli* with this "chimera" provided the first demonstration that genetic modification of *E. coli* was possible by a procedure that is now basic to recombinant DNA technology (Cohen *et al.*, 1973).

Since these initial studies were carried out, elegant cloning vectors, specific host cells, and methodologies have evolved. The most sophisticated systems for recombi-

nant DNA technology use *E. coli* and its plasmids and phages as components. However, "alternative" cloning systems have also been developed. Development of cloning systems in organisms other than *E. coli* appeared to reflect two interests. First, laboratory strains of *E. coli* are known to exchange genetic information with *E. coli* that are resident in the mammalian intestine. Hence, genes on plasmids in *E. coli* K12 are potentially transmissible to intestinal flora. Additionally, the *E. coli* cell envelope contains lipopolysaccharide, which causes endotoxin shock syndrome. Gene products produced in *E. coli* must therefore be rigorously purified from the lipopolysaccharide if they are to be used as pharmaceuticals. Both of the above concerns, voiced more than 10 years ago, have not caused health nor environmental difficulties in the practical use of *E. coli* for numerous gene cloning, expression, and production studies. This is due in large part to the care of the scientific community as well as the development of elaborate safeguards for recombinant DNA research designed to provide containment at the biological and physical levels. Indeed today, *E. coli* remains the workhorse for most recombinant DNA studies.

A second reason that alternative host-vector systems have evolved is based on important differences between microorganisms in specific functions. For example, *Streptomyces* species are known antibiotic producers, and it is probably simpler and more logical to modify antibiotics and their production by cloning into *Streptomyces* than to move antibiotic production into *E. coli* (Hopwood and Chater, 1984). Similarly, in order to study the cell division cycle in yeast by use of recombinant DNA technology, vector systems and transformation technology had to be developed for this lower eukaryote.

2. *BACILLUS SUBTILIS* AS A HOST FOR GENE CLONING

The *B. subtilis* system has emerged as the major prokaryotic alternative to cloning in *E. coli* for many reasons. The organism is not a primary pathogen, nor is it known to establish a stable association with humans. The nonpathogenic nature of *B. subtilis* is dramatically shown by the fact that spores of a very closely related species, *B. subtilis* var. *natto*, are eaten daily by millions of Japanese as part of a soybean fermentation product. It is acknowledged that *B. subtilis* produces endospores which are highly resistant to environmental stress. Thus, genes cloned in this bacterium can be maintained in a biologically active state for many years. However, nonreverting sporulation-deficient mutants exist, and many are highly transformable.

Aside from reasons of safety, *B. subtilis* has become a major tool for recombinant DNA studies largely because of three properties. *Bacillus* species secrete proteins into the culture medium (Priest, 1977; see Chapters 9 and 11). Therefore, it is possible to fuse heterologous coding sequences to the sequences encoding the signal peptide to achieve export of the product of a cloned gene. This is primarily of value because it provides an approach which, in theory, should allow extremely high-level production of a foreign protein without intracellular accumulation. In addition, *Bacillus* species undergo the developmental changes of endospore formation (Piggot and Coote, 1976; Losick, 1982; see Chapter 8). Analysis of this system by modern genetic methods requires a versatile and simple gene-cloning system. Lastly, *B. subtilis* has served as a model *Bacillus* for many years due to the identification

of a transformation system in the organism 30 years ago (Spizizen, 1958; see Chapter 4). As a consequence, *B. subtilis* is the most thoroughly characterized gram-positive bacterium in terms of both physiology and genetics. This point is adequately demonstrated by other chapters in this volume. Recent studies in many laboratories reveal that details of gene regulation and DNA replication differ significantly between the model gram-negative *E. coli* and gram positives such as *Staphylococcus* and *Bacillus* (Murray and Rabinowitz, 1982; Moran *et al.*, 1982). Thus, *B. subtilis* probably should be viewed as a model gram positive in which it is possible to conveniently analyze these differences.

Bacillus subtilis 168 is the strain typically used in nearly all genetic and physiological studies. This strain has long been known to lack detectable extra-chromosomal DNA (Carlton and Helinski, 1969). In the early 1970s, the attempts of one of us (P.S.L.) to transform several *E. coli* plasmids and a *Staphylococcus* plasmid, p1258, into *B. subtilis* 168 were unsuccessful. Therefore, strains of *B. subtilis* and members of the related species were screened for covalently cloned circular duplex DNA using dye-buoyant density gradient centrifugation (e.g., Lovett, 1973; Lovett and Burdick, 1973; Lovett and Bramucci, 1975; LeHagaret and Anagnostopoulos, 1977; Tanaka *et al.*, 1977). Hence, the first demonstration that plasmids exist in *Bacillus* was made in strains of *B. pumilus*, a species related to *B. subtilis* (Lovett, 1973; Lovett and Burdick, 1973). In this regard, it is important to recognize that several years prior to the detection of these plasmids, nearly one-third of the DNA in a strain of *B. megaterium* was shown to be in the form of CCC minicircles of heterogeneous size (Carlton and Helinski, 1969). However, the majority of these minicircles appeared to be the result of circularization of random chromosome fragments. Only recently have naturally occurring plasmids been identified in *B. megaterium* (Von Tersch and Carlton, 1983).

Because of the approach initially taken, the first plasmid successfully transformed into *B. subtilis* 168 originated in the *B. pumilus* strain ATCC 12140 (Lovett *et al.*, 1976). This plasmid, designated pPL10, is about 6 kilobase pairs and specifies a bacteriocin-like activity. pPL10 has been stably maintained in *B. subtilis* 168 for over a decade at a copy number of 10. Moreover, this plasmid can be eliminated by standard curing procedures that use ethidium bromide. Although pPL10 can easily be modified into a useful cloning vector, an observation by Ehrlich in 1977 dramatically enlarged the available plasmids for *B. subtilis* and altered the concept of relatedness among gram-positive bacteria (Ehrlich, 1977). Many small, high-copy, drug resistance plasmids known to reside in *Staphylococcus aureus* were successfully transformed into *B. subtilis* and many were stably maintained in this host. Subsequent to this observation, a tetracycline resistance plasmid, pBC16, was identified in *B. cereus* and introduced into *B. subtilis* (Bernhard *et al.*, 1978). Similarly, plasmids identified in *Streptococcus* can also be maintained and expressed in *B. subtilis* (Gryczan *et al.*, 1978). Since these early studies, drug resistance plasmids have been identified in both mesophilic and thermophilic gram-positive bacteria (e.g., Mahler and Halvorson, 1980; Bingham *et al.*, 1979; Lopez *et al.*, 1984).

3. PLASMIDS FOR *B. SUBTILIS*

Many plasmids have been successfully transformed into *B. subtilis* and are maintained stably in this species (see Table I for a partial list). However, most

Table I. Some Drug Resistance Plasmids Transformed into *B. subtilis* 168

Plasmid	Source	Mol. mass (MDa)	Drug resistance	Unique restriction sites ^a	References
pUB110	<i>S. aureus</i>	3	Neomycin/kanamycin	<i>EcoRI</i> , <i>BamHI</i> , <i>XbaI</i> <i>Bgl</i> III*	Gryczan <i>et al.</i> , 1978
pE194	<i>S. aureus</i>	2.4	Erythromycin	<i>HpaI</i> *, <i>BclI</i> *, <i>Hae</i> III*, <i>PstI</i> <i>XbaI</i>	Horinouchi and Weisblum, 1982a
pC194	<i>S. aureus</i>	2	Chloramphenicol	<i>Hae</i> III, <i>Hind</i> III, <i>HpaI</i>	Horinouchi and Weisblum, 1982b
pBC16	<i>B. cereus</i>	3	Tetracycline	<i>XbaI</i> , <i>EcoRI</i> , <i>Bam</i> HI, <i>HpaI</i>	Perkins and Youngman, 1983
pSA0501	<i>S. aureus</i>	2.7	Streptomycin	<i>EcoRI</i> , <i>Hind</i> III*, <i>XbaI</i>	Gryczan <i>et al.</i> , 1978
pSA2100	<i>S. aureus</i>	4.6	Streptomycin, chloram- phenicol	<i>EcoRI</i> *, <i>Hind</i> III*, <i>XbaI</i>	Gryczan <i>et al.</i> , 1978
pAM77	<i>Streptococcus</i> <i>sanguis</i>	4.5	Erythromycin		Yagi <i>et al.</i> , 1978
pT181	<i>S. aureus</i>	3	Tetracycline	<i>KpnI</i> *, <i>XbaI</i> , <i>PvuI</i>	Novick <i>et al.</i> , 1982 Khan and Novick, 1983

^aStarred sites are within drug resistance genes.

3.1. pUB110

This plasmid (Fig. 1; Appendix 3) was initially identified in *S. aureus* as a small, high-copy extrachromosomal element that specifies resistance to kanamycin and neomycin (Lacey and Chopra, 1974). Both resistances are due to a single gene on pUB110 that encodes the enzyme kanamycin (neomycin) nucleotidyltransferase (Gryczan *et al.*, 1980a; Sadaie *et al.*, 1980). The molecular mass of pUB110 determined by sucrose gradient centrifugation (2.8 MDa) and agarose gel electrophoresis (2.9–3.0 MDa) agrees well with the value of 4548 base pairs determined by nucleotide sequencing of the entire plasmid (Keggins *et al.*, 1978b; Gryczan *et al.*,

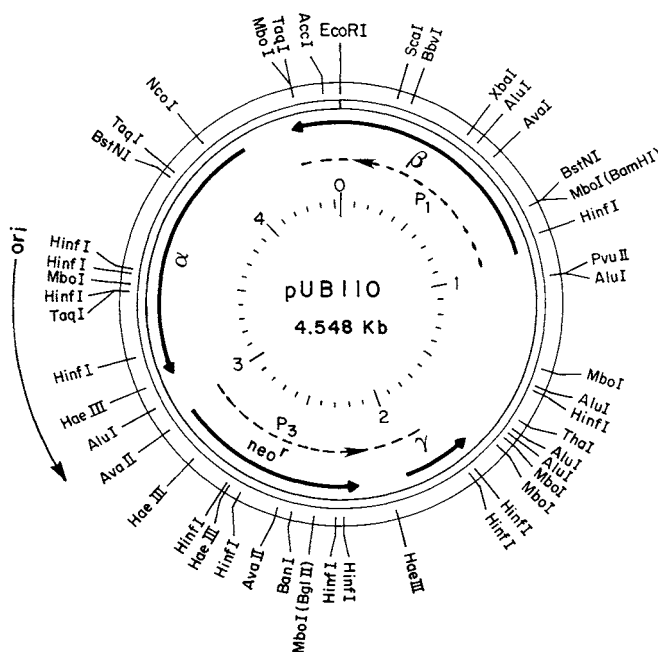


Figure 1. pUB110. This plasmid consists of 4548 base pairs as determined by nucleotide sequencing (McKenzie *et al.*, 1986, 1987; see Appendix pages A-110 through G-110). P₁ and P₃ are two transcript species identified by Curran and Stewart (1985) and Bruckner *et al.* (1984). Four open reading frames have been identified in the pUB110 sequence. One encodes the kanamycin/neomycin nucleotidyltransferase activity specified by pUB110. The figure shown here is redrawn from a diagram developed in the laboratory of N. Sueoka; see McKenzie *et al.*, 1986, 1987.

1978; McKenzie *et al.*, 1986, 1987). pUB110 is stably maintained in *B. subtilis* for at least 80 generations in the absence of selective pressure at a copy number estimated at about 50 per chromosome equivalent (Keggins *et al.*, 1978b). A plasmid initially detected in *B. cereus*, pBC16, has been shown to be largely homologous to pUB110 although pBC16 specifies tetracycline resistance and not neomycin/kanamycin resistance (Bernhard *et al.*, 1978; Polak and Novick, 1982). pBC16 has been transferred to a variety of strains related to *B. cereus* such as *B. anthracis* (Ruhfel *et al.*, 1984; Battisti *et al.*, 1985). Similarly, a plasmid detected in *B. stearothermophilus* also appears to be similar to pUB110 (Matsumura *et al.*, 1984). Lastly, the *Streptococcus* plasmid pAM α 1 appears to have resulted from the *in vivo* fusion of two replicons, one of which is nearly identical to pBC16 (Perkins and Youngman, 1983). Hence, pUB110 or pUB110-like plasmids seem to occur naturally in a wide range of gram-positive bacteria.

Electron microscopy of replicating pUB110 molecules that had been cut with *Eco*RI demonstrated a constant arm measuring about 22% of the pUB110 contour length and an arm of variable length (Scheer-Abramowitz *et al.*, 1981). As the replication loop increased, the length of the variable arm decreased. From these studies it has been inferred that pUB110 replicates from a fixed origin and replication proceeds in a unidirectional manner. The actual location of the replication origin on the pUB110 restriction map is based on the ability of deletion derivatives of pUB110 or pE104/pUB110 chimeras to replicate using the pUB110 origin. The presence of the pUB110 origin in pE194/pUB110 chimeras is inferred from the ability of the chimeras to replicate at 50°C, a temperature that is nonpermissive for pE194. The data suggest that pUB110 replication proceeds counterclockwise (Fig. 1).

Transcription studies of pUB110 have indicated that transcripts are synthesized in a counterclockwise direction. Both *in vivo* studies of a pUB110/pC194 chimera (pBD64) by S1 mapping and *in vitro* transcription studies using *B. subtilis* RNA polymerase have identified two major counterclockwise transcripts (Bruckner *et al.*, 1984; Curran and Stewart, 1985). One spans the unique *Bg*III site which marks the kanamycin/neomycin nucleotidyltransferase gene and the other extends from the *Pvu*II site toward *Bam*HI and *Eco*RI. The existence of pUB110 transcripts that pass through the *Eco*RI site in a counterclockwise direction was initially demonstrated by genetic studies. Insertion of the promoterless gene *cat-86*, which specifies chloramphenicol acetyltransferase (CAT), into the *Eco*RI site of pUB110 resulted in CAT activity only when the gene was oriented counterclockwise (Williams *et al.*, 1981a). Similarly, deletion analysis of the regulatory region of *erm* on a pUB110/pE194 chimera also appears to have been possible because of a pUB110 transcript that passes counterclockwise through the *Bam*HI and *Eco*RI sites (Hahn *et al.*, 1982).

In vivo analysis of proteins encoded by plasmids is conveniently approached by introducing plasmids into bacterial mutants that have an abnormal division plane. Such mutants produce small cells, termed minicells, which lack chromosome DNA but can harbor small plasmids. All *de novo* protein synthesis in minicells is directed by plasmid DNA. By use of a minicell-producing mutant of *B. subtilis* (Reeve *et al.*, 1973), pUB110 has been shown to specify three major proteins with molecular masses of 58,000, 38,000, and 11,000, and apparent minor proteins of 28,000 and 25,000 (Shivakumar *et al.*, 1979). The *Bg*III site in pUB110 is within the region

encoding kanamycin/neomycin resistance. Inserts into the *Bgl*III site alter the size of the 38,000-Da protein. Hence, this protein appears to be the kanamycin/neomycin nucleotidyltransferase. The molecular mass reported for the purified enzyme is 34,000 (Sadaie *et al.*, 1980).

3.2. pE194

This plasmid (Fig. 2; see Appendix 4) was originally detected in *S. aureus* and transformed into *B. subtilis* (Iordanescu, 1976; Gryczan and Dubnau, 1978). pE194 is 3728 base pairs in size and is maintained at a copy number of about 10 in *B. subtilis* at 32°C (Weisblum *et al.*, 1979; Horinouchi and Weisblum, 1982a). pE194 specifies resistance to macrolide, lincosamide, and streptogramin B (MLS) antibiotics. All resistances are due to a single gene, *erm*, whose expression is erythromycin-inducible and specifies an enzyme involved in methylating adenine residues in 23S rRNA (Shivakumar and Dubnau, 1981). The methylation reduces the affinity of the ribosome for MLS antibiotics (Lai and Weisblum, 1971; Lai *et al.*, 1973). The antibiotic tylosin does not induce drug resistance, although induction of *erm* expression with erythromycin confers resistance to tylosin. By selecting for pE194 derivatives that enable *B. subtilis* to grow on tylosin, two classes of plasmid mutations were identified (Weisblum *et al.*, 1979). One class results from mutations that allow constitutive *erm* expression and the other class results from mutations that elevate the copy number of pE194 up to 100 plasmids per cell. Both types of mutations have been mapped on the pE194 genome and further characterized (Horinouchi and Weisblum, 1980; Gryczan *et al.*, 1982; Shivakumar *et al.*, 1980).

pE194 exhibits two properties not shown by pUB110 or pC194. First, pE194 is naturally temperature-sensitive for replication. The plasmid replicates at 32°C, but at temperatures above 45°C (up to 50°C), replication ceases and the plasmid is lost from *B. subtilis*. A similar temperature-sensitive replication phenotype exists for the

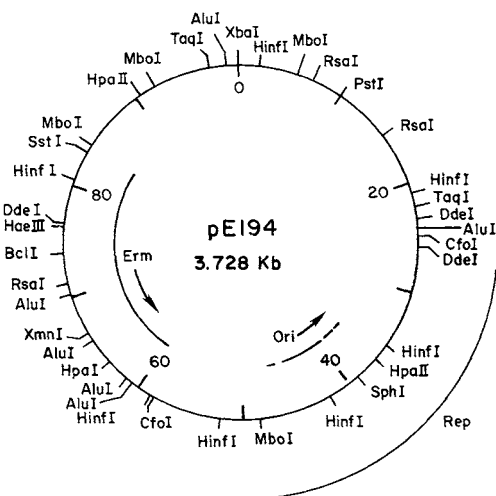


Figure 2. pE194. This plasmid consists of 3728 base pairs as determined by nucleotide sequencing (Horinouchi and Weisblum, 1982a; see Appendix pages A-E194 through E-C194). The representation of pE194 shown here is redrawn from Gryczan *et al.* (1982).

streptomycin resistance plasmid pSA0501. A second unusual feature of pE194 was detected when *B. subtilis* (pE194) was grown on erythromycin-containing media at temperatures which were nonpermissive for pE194. The result was integration of copies of pE194 into the host chromosome at a variety of sites (Hofemeister *et al.*, 1983).

3.3. pC194

A third plasmid commonly used in genetic and recombinant DNA experiments in *B. subtilis* is pC194 (Ehrlich, 1977, 1978; Fig. 3; Appendix 5). The complete nucleotide sequence of pC194 reveals that the plasmid consists of 2906 base pairs (Horinouchi and Weisblum, 1982b; Dagert *et al.*, 1984). pC194 is reported to be stably maintained in *B. subtilis* at a copy number of about 15 per cell, and pC194 specifies resistance to the antibiotic chloramphenicol due to the presence of a gene for chloramphenicol acetyltransferase (Byeon and Weisblum, 1984). The expression of this *cat* gene is inducible by chloramphenicol, as are all *cat* genes of gram-positive origin (Shaw, 1983). pC194 originated in *S. aureus*; however, it has been shown to provide functions which enable it to replicate not only in gram-positive bacteria but also in *E. coli* and *Saccharomyces* (Goze and Ehrlich, 1980; Goursoot *et al.*, 1982). Another unusual feature of pC194 is that this plasmid is incompatible with maintenance of the prophage of SPO2, a temperate *B. subtilis* phage (Marrero and Lovett, 1982). SPO2 lysogens which also contain pC194 rapidly lose the SPO2 prophage. This SPO2 incompatibility has not been observed with pE194 or pUB110. A third unusual aspect of pC194 involves its ability to enhance M13 transduction of pBR322 when the two plasmids are joined. pC194 has been shown to actively participate in the enhanced transducibility because mutations which inactivate either of two open reading frames on pC194 which are essential to replication in *B. subtilis* block the enhancement of transduction (Dagert *et al.*, 1984; Dagert and Ehrlich, 1984).

Insertion of pC194 into *B. subtilis* minicells revealed that the plasmid specifies two proteins of 22,000 and 11,000 Da (Shivakumar *et al.*, 1979). The 22,000-Da peptide is inducible with chloramphenicol and therefore is the chloramphenicol acetyltransferase subunit. This value is only slightly below the predicted molecular size for the protein based on the sequence of the *cat* gene.

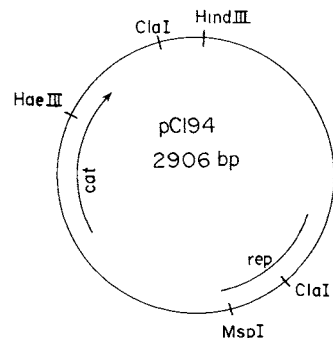


Figure 3. pC194. This plasmid consists of 2906 base pairs as determined by nucleotide sequencing (Horinouchi and Weisblum, 1982b; Dagert *et al.*, 1984; see Appendix A-C194 through E-C194).

pC194 is currently being studied for its replication and segregation properties (e.g., Alonso and Trautner, 1985). Although this is beyond the scope of this chapter, these studies will contribute to understanding this novel small genome.

3.4. Sites Available for Gene Cloning in pUB110, pE194, and pC194

In theory, non-self-replicating DNA fragments can be cloned into plasmids at restriction sites located in regions uninvolved in plasmid replication. In the case of the 4.5-kilobase plasmid pUB110, this region includes the 3-kilobase portion extending clockwise from *EcoRI* through the kanamycin/neomycin resistance gene. Unique restriction sites in this region include *EcoRI*, *BamHI*, *XbaI*, *PvuII*, and *BglII*. The *BglII* site is within the kanamycin/neomycin resistance gene. Hence, fragments inserted into *BglII* inactivate the drug resistance specified by pUB110, although replication is unimpaired.

The region on pC194 apparently involved in replication is an approximately 1-kilobase pair region extending from coordinates 1.5 to 2.5 kilobases on the pC194 map (Fig. 3). Accordingly, a restriction site which is unique in pC194 and available for insertion of non-self-replicating DNA fragments is the *HindIII* site. Inserts at *HindIII* do not inactivate the chloramphenicol resistance gene specified by pC194.

pE194 replication is determined by sequences within a 1.3-kilobase pair *TaqI* fragment, designated *TaqIB*. Unique restriction sites in pE194 outside of this fragment include *HpaI*, *BclI*, *PstI*, *XbaI*, *HaeIII*, *AccI*, and *BstI*. However, the *erm* gene (encoding erythromycin resistance) is inactivated by inserts at *HpaI*, *HaeIII*, and *BclI*.

3.5. Plasmid Amplification in *B. subtilis*

Chloramphenicol amplification of ColE1-like plasmids in *E. coli* has been extensively used to increase the amount of plasmid DNA, and hence cloned DNA sequences, recovered from small volumes of cells. Plasmids whose replication is similar to ColE1, and are therefore amplifiable when protein synthesis is blocked, have not yet been detected among the many plasmids known to replicate in *B. subtilis*. However, several plasmids such as pUB110 have been shown to replicate selectively in *B. subtilis* when chromosome replication is blocked by use of specific temperature-sensitive DNA replication mutants (Shivakumar and Dubnau, 1978). This novel approach to plasmid amplification has not yet been used to any significant extent for two probable reasons. First, many plasmids that replicate in *B. subtilis* are already present at very high copy number. Thus, amplification apparently has not been considered as a key advantage for the isolation of DNA. Second, *B. subtilis* cloning has largely been used in small-scale studies where the genetics of the construction is of primary interest rather than the isolation of large amounts of the DNA.

3.6. Vectors Constructed by Fusing "Gram-positive" Plasmids

In order to construct plasmid vectors that specify two or more drug resistance traits, various plasmids have been fused *in vitro* (Ehrlich, 1978; Gryczan and Dub-

Table II. Plasmids Constructed by *in Vitro* Ligation of Two Plasmids

Plasmid	Size (MDa)	Method of construction	Drug resistance	Host ^a	References
pBD6	5.8	Fusion of pUB110 and pSA0501 at <i>Xba</i> I	Neomycin/kanamycin, streptomycin	B	Gryczan <i>et al.</i> , 1980b
pBD8	6	Fusion of pUB110 and pSA2100 at <i>Xba</i> I	Neomycin/kanamycin, streptomycin, chloramphenicol	B	Gryczan <i>et al.</i> , 1980b
pBD64	3.2	Fusion of pUB110 and pC194 at <i>Hpa</i> II plus an <i>in vivo</i> spontaneous deletion	Neomycin/kanamycin, chloramphenicol	B	Gryczan <i>et al.</i> , 1980b
pHV14	4.6	Fusion of pBR322 and pC194 at <i>Hind</i> III	Chloramphenicol in <i>Bacillus</i> ; chloramphenicol and ampicillin in <i>E. coli</i>	B/E	Ehrlich, 1978

^aB and E indicate ability to replicate in *Bacillus* only, or *Bacillus* and *E. coli*.

nau, 1978; Gryczan *et al.*, 1980b). When one of the drug resistance genes contains a unique restriction enzyme site, fragment insertion into this site is accompanied by inactivation of the drug resistance trait. Table II presents a list of example plasmids constructed by fusions between pUB110 (Km/Nm), pC194 (Cm), pSA0501 (Sm), or pSA2100. pSA501 is temperature-sensitive for replication and therefore recombinants which depend on this replicon are capable of replication at 32°C but not at 50°C. pBD64 is the result of fusion of pUB110 and pC194 followed by an *in vivo* deletion event that removed much of the pC194 sequences including the *Hind*III site (Gryczan *et al.*, 1980b).

3.7. Absence of Low-Copy Plasmid Vectors for Gene Cloning

The above plasmid vectors are maintained in *B. subtilis* at copy numbers of probably 10 or more. This is known for plasmids pUB110, pE194, and pC194 and is inferred for the constructions based on these plasmids. Plasmid cloning vectors suitable for stable maintenance of cloned genes in an extrachromosomal state at a copy number approaching one per chromosome have not been described. Such vectors are likely to be developed using replicons derived from single-copy cryptic plasmids (e.g., Lovett and Bramucci, 1975). In the absence of such vectors, alternative methods for maintaining cloned genes at low copy numbers involve insertion into the host chromosome by any of several methods to be outlined later.

3.8. Shuttle Vectors

Plasmids constructed to allow replication in two or more unrelated organisms have been referred to as shuttle vectors, bridge vectors, or bifunctional replicons. Thus, plasmids have been developed that can replicate in *E. coli* and yeast or in *E.*

coli and animal cells. Most plasmids do not naturally act as shuttle vectors because, for example, plasmid sequences that permit replication in *E. coli* seem to be inadequate for replication in yeast. Plasmids from *E. coli* do not replicate in *B. subtilis* and the reverse is generally true, with a specific exception: pC194 can reportedly replicate in both *E. coli* and *B. subtilis* (Goze and Ehrlich, 1980). The first shuttle plasmids capable of replication in *B. subtilis* and *E. coli* resulted from *in vitro* fusion of well-characterized *E. coli* plasmids such as pBR322 with various plasmids that replicate in *B. subtilis* (Ehrlich, 1978; Kreft *et al.*, 1978; Table 2). These plasmids provided the first direct evidence that *B. subtilis* was not capable of expressing genes that originate in *E. coli*. For example, when pUB110 and pBR322 are fused at their unique *Eco*RI sites, the resulting shuttle vector expresses in *E. coli* the three drug markers neomycin resistance, ampicillin resistance, and tetracycline resistance. When introduced into *B. subtilis* the same plasmid expresses only neomycin resistance efficiently, although tetracycline resistance is weakly expressed. The value of *B. subtilis*/*E. coli* shuttle vectors is largely seen in their use for cloning *B. subtilis* restriction fragments in *E. coli* and the subsequent return of the recombinant molecules to *B. subtilis*.

3.9. Plasmid Copy Number and Stability

Virtually all plasmids that are currently used for gene-cloning studies in *B. subtilis* are multicopy replicons maintained in cells at 10 copies or more per chromosome equivalent (see Chapter 7 and Appendix 7 for copy number methods). The actual stability with which plasmids are maintained in *B. subtilis* depends on several conditions. For example, pUB110 is stably maintained in *B. subtilis* at 50 copies/chromosome, regardless of whether or not host cells are grown in the presence of neomycin or kanamycin. However, cloning large fragments of DNA into pUB110-based replicons can decrease both copy number and plasmid stability (see Chapter 7). For example, pPL703 is a pUB110-based replicon containing the chloramphenicol resistance gene *cat-86* (Mongkolsuk *et al.*, 1983). Derivatives of pPL703 in which *lacZ* has replaced *cat-86* are stably maintained in *B. subtilis* only as long as selection for the plasmid is maintained (Mongkolsuk *et al.*, 1984). By comparison, pPL703 itself is maintained stably without selection.

Plasmid pAM77 is a small erythromycin resistance plasmid that replicates in *B. subtilis* (Yagi *et al.*, 1978). pAM77 is very unstable in *B. subtilis* unless drug selection for the plasmid is maintained. Plasmids such as pC194 or pE194 are relatively stably maintained in *B. subtilis*, although pE194 is temperature-sensitive for replication above 45°C. However, during experimental manipulations with pC194 or pE194 we have isolated clones that have "spontaneously" lost the plasmids, whereas this is generally not seen with pUB110.

Plasmid stability also refers to resistance of plasmids to internal rearrangements, including deletions (see Chapter 7). Although plasmids that have been transformed into *B. subtilis* do not seem to become physically rearranged, the use of plasmids for cloning DNA fragments does, in certain instances, promote the formation of deletions of varying sizes (e.g., Keggins *et al.*, 1978a,b). Deletion formation has been occasionally observed when pBR322 is fused to replicons such as pC194, when two gram-positive plasmids are joined, or when non-self-replicating restriction fragments are cloned into plasmids (Primrose and Ehrlich, 1981). Deletions have

been shown to occur in recombinant DNA molecules introduced into Rec⁺ or Rec⁻ strains (Keggins *et al.*, 1978b; Uhlen *et al.*, 1981). The basis for these deletions remains unknown, although recent studies implicate direct-repeat sequences at the boundaries of the deletions (Hahn and Dubnau, 1985).

4. PLASMID TRANSFER IN *B. SUBTILIS*

4.1. Transformation of Competent Cells

Many strains of *B. subtilis* are naturally capable of binding DNA fragments and transporting the fragments to an intracellular location (Spizizen, 1958; Dubnau, 1982; see Chapter 4). This physiological state is referred to as competence, and occurs within a small percentage of the cells in a culture shortly after cessation of exponential growth. Competent cells of *B. subtilis* can be transformed with plasmid DNA, which is not homologous with the *B. subtilis* chromosome. This was first shown using the *B. pumilus* plasmid pPL10 (Lovett *et al.*, 1976). Covalently closed circular DNA added to competent cells was taken up and maintained extra-chromosomally at a fixed copy number. Comparison of the transformed plasmid with the original untransformed plasmid indicated that no change had occurred during entry into *B. subtilis*. Subsequent analysis of plasmid transformation in several laboratories has shown that competence for plasmid DNA and chromosomal DNA occurs at the same growth stage, and the uptake kinetics for both types of DNA are similar (Contente and Dubnau, 1979; deVos *et al.*, 1981). However, plasmid transformation is inefficient, requiring a DNA input equivalent of about 1000 plasmids to achieve a single plasmid-containing transformant. This seems not to be due to a requirement for recombination among incoming plasmids since about equal efficiencies of transformation can be obtained with Rec⁺ and Rec⁻ cells (see below). A remarkable finding by Canosi *et al.* (1978) explained the basis for the inefficiency of plasmid transformation and has in turn stimulated the development of models to explain plasmid transformation in *B. subtilis*. In the initial study, Canosi *et al.* (1978) used the chloramphenicol resistance plasmid pC194. It was shown that while pC194 could be transformed into competent *B. subtilis*, stable plasmid-containing transformants only resulted when the donor plasmid was a trimer or higher multimeric form. No significant transformation resulted from introduction of monomers or dimers (Mottes *et al.*, 1979). Since monomers make up more than 90% of the DNA in purified plasmid preparations, this is the apparent explanation for the inefficiency of plasmid transformation. A likely explanation for the requirement that transforming plasmids must be multimers is based on the observation that donor linear duplex DNA fragments actually enter *B. subtilis* as single-stranded molecules. During entry one strand is degraded to acid-soluble material. Accordingly, if a monomeric plasmid form contacts *B. subtilis*, it is thought to be converted to a linear form, which is then subjected to the loss of one strand during entry. It is probable that the ends of the single-stranded form which enters are subjected to nibbling by single-stranded specific exonucleases. Since there is nothing in the cell genome to which a plasmid is homologous, no heteroduplex is formed and therefore the plasmid is not converted to a duplex form. If, on the other hand, a circular multimeric plasmid resulting from direct repeats of the

monomeric plasmid form is added to *B. subtilis* cells, the decay process would allow entry of complementary single strands. This single-stranded circle can be converted to a duplex circle by repair synthesis with DNA polymerase I and ligase.

The necessity of using multimeric plasmids for transforming *B. subtilis* provides a major obstacle when competent cells are used as recipient for cloning genes; the act of restriction enzyme digestion and subsequent ligation (with or without target DNA fragments) generates primarily monomeric plasmid forms. Therefore various strategies have been developed to overcome this difficulty (see Section 7). One alternative is the use of a protoplast transformation system for gene cloning rather than competent cells.

4.2. Transformation of Protoplasts

Protoplasts of *B. subtilis* are prepared by lysozyme treatment of cells in media supplemented with osmotic stabilizers such as sucrose, sodium chloride, or succinate. Chang and Cohen (1979) demonstrated that protoplasts of *B. subtilis* can be transformed with plasmids in the presence of polyethylene glycol. Subsequent regeneration of the cell wall of the transformed protoplast allows selection of the plasmid-containing transformants. The transformation efficiency of protoplasts by plasmids is much higher than is obtained using competent cells. This is probably due to the observation that monomeric plasmids can transform protoplasts. Therefore, protoplast transformation seems to eliminate the major problem associated with plasmid transformation of competent cells. Accordingly, the process of shotgun cloning of genes in plasmids would seem to be best approached by use of the protoplast transformation system. Unfortunately, this technique has not been as widely used as one might expect. Although the reasons for this lack of use are not totally clear, there are manipulations associated with the protoplast transformation system that are not encountered when competent cells are used. First, competent cells can be readily generated by simply growing *B. subtilis* cells in Spizizen minimal medium, and competent cells can be frozen and stored for several months prior to use. Protoplasts are prepared by lysozyme treatment of cells in a stabilized medium, and these do not survive freezing. Second, the medium that allows high-frequency regeneration of cells from protoplasts is nutritionally complex. Therefore, direct selection for nutritional markers is, in most cases, not possible. Thus, a minimal medium that allows high-efficiency cell wall regeneration would be very useful.

Strains of *B. subtilis* and one strain of *B. licheniformis* (Thorne and Stull, 1966) can become competent for transformation when grown under a specific regimen. However, many strains of *B. licheniformis*, *B. pumilus*, *B. thuringiensis*, *B. megaterium*, and other species are not known to become naturally competent. Significantly, protoplasts of *B. thuringiensis*, *B. megaterium*, *B. pumilus*, and *B. licheniformis* have been successfully transformed with plasmid DNA (Brown and Carlton, 1980; Martin *et al.*, 1981; Lovett, unpublished). Hence, the protoplast transformation system greatly extends the *Bacillus* species into which plasmids can be transformed.

4.3. Plasmid Transfer by Transduction

Plasmid transfer among bacteria by the use of transducing phage provided a valuable method for plasmid study in *E. coli* and *S. aureus*. Since the first plasmids in

Bacillus species were detected in strains of *B. pumilus*, plasmid transfer by transduction was initially demonstrated in members of this species. Plasmid pPL10 specifies bacteriocin activity in *B. pumilus* strains (Lovett *et al.*, 1976). This plasmid was transferred among strains of *B. pumilus* by the generalized transducing phage PBP1 (Lovett, 1972). The host range for PBP1 seems limited to *B. pumilus* strains, whereas the phage PBS1 has a much broader host range and has been used to transfer plasmids such as pUB110 and pC194 between *B. subtilis* and *B. pumilus* (Keggins *et al.*, 1978b). Similarly, the phage SP15 infects *B. subtilis* strains and strains of *B. licheniformis* (Taylor and Thorne, 1963; Hemphill and Whitely, 1975). Hence, SP15 can be used to move plasmids between these species. Transducing phages also exist for members of the *B. cereus* group of bacteria (Thorne, 1968) and these have been successfully used to transfer pBC16 among members of this bacterial group (Ruhfel *et al.*, 1984). Based on these and other studies (Canosi *et al.*, 1982), it is likely that any phage capable of generalized transduction can transduce plasmids, but at varying efficiencies (e.g., Bramucci and Lovett, 1976).

Two of the most well-studied temperate phages for *B. subtilis* are SPO2 and ϕ 105 (Hemphill and Whitely, 1975). Neither of these phages can perform transduction of any of several small drug resistance plasmids. However, when fragments of ϕ 105 DNA are cloned into a plasmid, the resulting chimeras become transducible by ϕ 105 at frequencies ranging from 10^{-5} to 10^{-6} transductants per plaque-forming unit (Marrero and Lovett, 1980). Similar results have been obtained with SPO2, SPPI, and the *Salmonella* temperate phage P22 (Marrero and Lovett, 1980; Orbach and Jackson, 1982; Deichelbohrer *et al.*, 1985). It is thought that insertion of, for example, ϕ 105 DNA into a plasmid provides a region of homology between the infecting phage and the resident plasmid. Homologous recombination between phage and plasmid can therefore occur allowing the generation of plasmid-phage recombinants which are packaged into phage heads. When such phage particles are used to infect a recipient host the plasmid is thought to excise and replicate independently.

A second mechanism that allows ϕ 105 and SPO2 to transduce plasmids requires cloning the cohesive ends of the phages into plasmids (Marrero and Lovett, 1981; Flock, 1983). The cohesive ends of temperate phages, genetic designation *cos*, are directly involved in packaging of phage DNA into heads during viral morphogenesis. The *cos* sites cloned from SPO2 or ϕ 105 DNA into plasmids confer on the plasmid exceedingly high transducibility by the respective phage. For example, the *cos* site of SPO2 occurs within a 1.6M-Da *Eco*RI fragment. Insertion of this fragment into plasmids pUB110 or pC194, molecular masses 3 and 2M Da, respectively, allows their transfer by SPO2 at a frequency of about one transductant per 100 plaque-forming particles. In absolute terms, this allows the generation of phage lysates that contain 10^8 – 10^9 transducing particles per milliliter (Marrero and Lovett, 1981). Hence this system offers the highest efficiency transfer of plasmids in *B. subtilis*.

A unique feature of SPO2 particles that contain *cos* plasmids is the resistance of the transducing activity to ultraviolet irradiation. Irradiation of lysates sufficient to inactivate more than 90% of the plaque-forming activity has no effect on the transducing activity. In addition, SPO2 phage particles that carry pUB110-*cos* plasmids have a lower buoyant density than infectious particles. Both of these properties (Marrero and Lovett, 1981) reflect the molecular form in which SPO2 *cos* plasmids are maintained in SPO2. pUB110 *cos* is carried by SPO2 as a linear multimer whose

size approximates the mass of SPO2 infectious DNA (~30M Da. Since pUB110-*cos* is about 4.6M Da, the linear multimer probably contains seven linear plasmids joined head to tail. Each transducing particle carries only the multimer and not infectious DNA. The difference in buoyant density between transducing particles and infectious particles is due to the different densities of pUB110 and SPO2 DNA. The resistance of the SPO2 *cos* transduction to ultraviolet light is not seen when the transduction recipient is recombination-deficient and is likely to be the result of recombination among the monomers forming functional monomeric plasmids (Marrero and Lovett, 1981).

SPO2 particles can adsorb and inject DNA into many *Bacillus* species that do not allow productive infection of SPO2. Therefore, insertion of SPO2 *cos* into plasmids allows their transfer to certain *Bacillus* species which are not transformable and are not transduced by the standard phages (Marrero *et al.*, 1984).

4.4. Plasmid Transfer by Co-Conjugation

Certain plasmids, apparently those related to pUB110, can be mobilized by a conjugal plasmid pLS20, originally identified in *B. subtilis* var. *natto* (Koehler and Thorne, 1987). pLS20 has been shown to mobilize pBC16 and pUB110 by a donation mechanism. Although many details of the conjugal mechanism remain to be elucidated, the system may prove useful for specific genetic manipulations.

5. RECOMBINATION-DEFICIENT MUTANTS OF *B. SUBTILIS*

Mutants of *E. coli* which are deficient in homologous recombination are hypersensitive to ultraviolet light and to radiomimetic drugs. Accordingly, recombination-deficient mutants of *B. subtilis* were sought by testing the ability of many mitomycin C-hypersensitive mutants to be transformed. By this general approach approximately 12 genetic loci have been identified which are involved in various aspects of the recombination pathway(s) (Dubnau *et al.*, 1973; Dubnau and Cirigliano, 1974; Sadaie and Kada, 1976). The genetic designations of several *rec* mutations vary between the various laboratories involved in the isolation.

Only one class of Rec mutant, *recE4*, has been used extensively in gene-cloning studies. The *recE* gene in *B. subtilis* is thought to be analogous to the *recA* gene in *E. coli* for several reasons: (1) *recE* mutations completely block homologous recombination, whereas recombinational blocks caused by the other *rec* mutations seem less complete; (2) *recE* mutations block induction of prophages of SPO2 and ϕ 105 in an analogous manner to *recA* mutations; (3) the recombination deficiency in a *recE* mutant was overcome by cloning into *B. subtilis* a functioning *recA* gene from *E. coli* (DeVos *et al.*, 1984); and (4) antibodies directed against the *E. coli* RecA protein react(s) with a protein in *B. subtilis* and allowed the purification of this "RecA-like" protein (Lovett and Roberts, 1985). This RecA-like protein is reportedly absent from *recE* mutants.

Since the *recE* gene product is essential for homologous recombination, chromosome markers cannot be transformed or transduced into *recE* mutants if their maintenance requires recombination with the recipient chromosome. Plasmid

transformation is, however, seemingly unaffected by the presence of a *recE* mutation in the recipient (Keggins *et al.*, 1978a,b). Hence, *recE* mutants allow the extrachromosomal maintenance of fragments of *B. subtilis* DNA cloned into plasmid vectors. The use of *recE* recipients for cloning homologous DNA into *B. subtilis* was first reported by Keggins *et al.* (1978b). In these studies, a fragment of *B. subtilis* DNA spanning the *trpC* gene was cloned into pUB110 and introduced into BD224, a *trpC2*, *recE4* mutant. This plasmid did not recombine with the host chromosome but did complement the *trpC2* mutation. Loss of the plasmid restored the requirement for tryptophan.

recE4 also blocks recombination between plasmids which carry homologous DNA fragments. To demonstrate this, Keggins *et al.* (1978a) used the compatible, unrelated plasmids pUB110 and pPL576. Into each plasmid was inserted a small DNA fragment that spanned the *B. pumilus trpC* gene. pUB110 harbored mutant version of the *trpC* gene and pPL576 harbored a wild-type gene. When these plasmids were introduced into a recombination-proficient *B. subtilis* strain, the mutant *trpC* allele on pUB110 was repaired by recombination at very high frequency. This was detected by cotransformation of pUB110 and *trpC*⁺. In contrast, when the two plasmids were inserted into a *recE4*-containing strain, no recombination between the plasmids was detected.

recE in *B. subtilis* and *recA* in *E. coli* are probably analogous genes. *recE* was recently cloned and the reported studies suggest that the protein product is related to the *E. coli recA* protein (Marrero and Yasbin, 1988). For most purposes, therefore, *recE* mutants can be used in the same manner as *recA* mutants. One possible exception has emerged. *recA* mutants allow the stable inheritance of plasmid multimers introduced into *E. coli* (Potter and Dressler, 1977). Apparently, the *recA* gene product is necessary for converting multimers to monomers. As noted earlier (Section 4.1), competent *B. subtilis* cells seem to be transformed only by multimeric plasmids. However, when competent *recE* strains are transformed with plasmids, the predominant plasmid form in the transformants is a monomer. Therefore, it is likely that another enzyme is present in *recE* strains which converts multimers to monomers.

6. RESTRICTION AND MODIFICATION IN *B. SUBTILIS*

Enzymic restriction in bacteria is due to endonucleases that cleave susceptible DNA. Resistance of DNA to cleavage is due either to the absence of the recognition and cleavage sites on the DNA or to modifications of susceptible sites which have rendered them insensitive to cleavage. Modification of DNA to a form that is insensitive to cleavage generally involves a unique pattern of methylation of bases by a specific modification (methylating) enzyme. Restriction–modification systems in *E. coli* reduce the replication efficiency of foreign (unmodified) DNA because the incoming duplex DNA is generally cleaved before it is completely modified. Thus in *E. coli* it is of value to use restriction-negative mutants for cloning foreign DNA. Most restriction enzymes cleave only duplex DNA, not single-stranded DNA. Exceptions to this rule exist, such as *HaeIII*, which cleaves both duplex and single-stranded DNA. Six different restriction–modification systems have been identified in various strains of *B. subtilis* (Trautner *et al.*, 1974; Shibata and Ando, 1974; Bron

and Murray, 1975; Shibata *et al.*, 1976, 1979; Saito *et al.*, 1979; Ikawa *et al.*, 1980, 1981; Fucik *et al.*, 1982). However, *B. subtilis* strain 168 contains only the system designated *BsuM*, which includes a restriction enzyme that is an isoschizomer to *Xho* I (Jentsch, 1983). The restriction system has been shown to be effective in reducing the efficiency of plaque formation by bacteriophages such as ϕ 105, whose DNA is unmodified or incorrectly modified. Moreover, bacterial mutants deficient in restriction–modification have been isolated and these are highly transformable (Tanaka and Sakaguchi, 1978). However, these restriction–modification–defective mutants have not been widely used in gene cloning in *B. subtilis*, largely because no major problem involving restriction of foreign DNA has been encountered.

Most gene-cloning studies that involve introducing foreign DNA in *B. subtilis* (e.g., DNA from *E. coli*) have used competent cells as the transformation recipient. Competent cells degrade incoming circular duplex molecules first to linear forms and then to single-stranded forms. Hence the molecule that actually enters competent cells is largely single-stranded and presumably insensitive to restriction. Conversion of the largely single-stranded DNA to a duplex form is probably achieved by DNA synthesis and concomitant modification. In contrast monomeric plasmids transforming *B. subtilis* protoplasts are likely to be entering as a DNA duplex. It would be expected that unmodified plasmids transformed into *B. subtilis* protoplasts would be susceptible to the host restriction enzyme. Two studies, however, suggest that transformation of competent, restriction-positive strains of *B. subtilis* with unmodified plasmid DNA does result in a significant decrease in the number of transformants recovered (Tanaka, 1979; Prozorov *et al.*, 1980). Since transformation of chromosome markers is relatively insensitive to restriction (Bron *et al.*, 1980), it seems that transformation events involving plasmids must result in a sensitive duplex state at some stage of the process. Similar studies of plasmid transformation in restriction-positive and negative protoplasts have not been reported.

7. METHODS FOR GENE CLONING USING PLASMID VECTORS

Methodology for cloning genes in *B. subtilis* varies according to the transformation host used to recover the initial recombinant plasmids.

7.1. *B. subtilis* Competent Cells as Cloning Host

Although it is possible that monomers can transform at very low efficiency, it is clear that the majority of plasmid transformants result from introduction of trimers or higher multimeric structures (see Section 4.1). Since the process of gene cloning involves digestion of vector and target DNA with a restriction enzyme that generally cuts the vector once, this reduces multimers to linear monomers. Thus, ligation of cleaved plasmid generally would be expected to produce predominantly circular monomers, with or without an inserted piece of target DNA. The consequence is that the act of gene cloning, following procedures developed for *E. coli*, greatly diminishes the presence of multimers. Despite this, cloning by this type of procedure has been successfully used to recover plasmids fused *in vitro* and to clone chromosomal genes, promoters, and phage DNA fragments. This may result from

the fact that in such cloning, vector concentrations are relatively high, possibly generating *in vitro* formation of multimers at varying efficiencies. Thus, a successfully transforming recombinant plasmid constructed *in vitro* may contain one fragment of target DNA ligated to three plasmid molecules.

In the first study of cloning chromosomal genes Keggins *et al.* (1978b) used standard *E. coli* methodology. Fragments of *Bacillus* DNA containing *trp* genes were cloned in pUB110 using *B. subtilis* competent cells as transformation recipient. Each transformant identified that contained a pUB110-*trp* recombinant invariably also contained copies of pUB110. The two forms of pUB110, the *trp* recombinant and the native plasmid, were easily separated by isolating DNA from the initial transformant, and retransforming for the Trp⁺ phenotype. Since this phenomenon has virtually always been observed in numerous such clonings, using Rec⁺ or Rec⁻ cells as recipient, it suggests that the *in vitro*-generated recombinant could be a multimeric plasmid form. Furthermore, the occurrence of both vector and recombinant in transformants makes it impossible to use inactivation of a plasmid marker as an indication that a DNA fragment has been inserted into the vector. For example, in a study by Williams *et al.* (1981b) a segment of the *E. coli trp* operon was fused in-frame within the coding sequence of the *cat-86* gene, specifying chloramphenicol acetyltransferase, on a plasmid pPL608. Although the insertion of the *E. coli* DNA into *cat-86* inactivated the gene, all transformants of *B. subtilis* that contained the *trp*-pPL608 recombinant were chloramphenicol-resistant because copies of pPL608 were also present in the cells.

In current cloning experiments, we generally mix 0.5 µg/ml of target DNA with 2 µg of plasmid DNA. Each is cut with an enzyme that cleaves the plasmid once. The DNA is ligated and transformed into competent cells. Transformants are directly plated to select for the desired gene. This general method has permitted us to clone chromosomal genes, fragments of ϕ105 and SPO2 phage DNA, and we commonly use this method to clone promoters. However, the method is highly inefficient relative to cloning in *E. coli* using the identical procedure.

A second cloning method was developed that allows the cloning of heterologous DNA fragments in *B. subtilis*. The approach involves gene cloning by recombination between a plasmid resident in the transformation recipient and a donor plasmid which contains a cloned fragment (Gryczan *et al.*, 1980a). The rationale for this cloning method is that only multimeric plasmid DNA can apparently give rise to a stable plasmid-containing transformant. This is likely due to the conversion of transforming duplex DNA to a single-stranded form during entry. Monomeric, single-stranded plasmid DNA apparently cannot be converted to a circular duplex, whereas multimeric, single-stranded DNA can contain regions of intramolecular homology and therefore can be cyclized. Since monomeric plasmid DNA is thought to enter the cell in a single-stranded form, it could be recovered in the host as a stable recombinant if a region of homology were present. This is dependent on the generalized recombination, and cloning by this approach cannot be performed in a *recE*-deficient host. As a consequence only heterologous fragments (DNA that does not recombine with the *B. subtilis* chromosome) can be recovered by the procedure.

Gene cloning by the "resident plasmid technique" is performed by use of a transformation recipient that contains a plasmid, such as pUB110, which is largely homologous with a plasmid, such as pBD64, that is to be used for cloning heterologous fragments. In a typical experiment, pBD64 is digested with *Bgl*II which cuts

into the Km/Nm gene located in the pUB110 portion of pBD64. *Bacillus licheniformis* DNA (which is largely nonhomologous with *B. subtilis* DNA) can be cut with an enzyme that produces fragments with single-stranded ends that can anneal to the ends generated by *Bgl*II cleavage; such ends are generated by cleaving with *Bcl*II, *Bgl*II, *Bam*HI, *Mbo*I, or *Sau*3A. The cut pBD64 and cut *B. licheniformis* DNA are ligated with T4 DNA ligase and transformed into competent *B. subtilis* cells that contain pUB110. If a *trp* gene is to be cloned from the *B. licheniformis* DNA, the *B. subtilis* recipient should contain a mutation in the corresponding *trp* gene. After transformation the recipient is plated on a medium lacking tryptophan. The resulting Trp⁺ transformants contain a plasmid indistinguishable from pBD64 that harbors an insert at *Bgl*II. This method has been successfully used by many laboratories for cloning heterologous DNA fragments into *B. subtilis*. We have found that transformants remain neomycin-resistant because copies of pUB110 remain in the cell. This presumably is due to the involvement of only a few resident pUB110 molecules in the recombination event. Although recombinant molecules can be separated from the resident pUB110 molecules, it has not been possible to use insertional inactivation to detect DNA inserts. Perhaps a lower copy plasmid would be useful in this regard.

7.2. *B. subtilis* Protoplasts as Cloning Host

Bacillus subtilis protoplasts are reportedly transformable with plasmid monomers (Gryczan *et al.*, 1980a). Thus, plasmid DNA and target DNA cut with a common restriction enzyme, ligated and transformed into protoplasts, should in theory allow recovery of recombinant plasmids. The medium used to regenerate protoplasts, DM3, is a complex medium (Chang and Cohen, 1979) and does not permit selection of, for example, nutritional markers. It is possible, however, to detect cloned genes that encode extracellular degradative enzymes using DM3 media. Incorporation of starch or casein in this complex medium permitted the direct identification of amylase and protease genes cloned in *B. subtilis* protoplasts (Corfield *et al.*, 1984).

7.3. *E. coli* as Cloning Host

Fragments of *B. subtilis* DNA can be cloned directly into *E. coli* strain HB101 or JM103 using any of several cloning vectors such as pBR322. Cloning in *E. coli* allows the use of plasmid DNA that has been linearized with a restriction enzyme and treated with bacterial or calf alkaline phosphatase to block self-ligation. Therefore in *E. coli* the majority of plasmid-containing transformants can be forced to contain a recombinant molecule. Furthermore, the technique of insertional inactivation of plasmid drug resistance or sugar utilization genes provides a rapid test for the presence of a cloned DNA fragment. These and other techniques used in cloning in *E. coli* are documented elsewhere (Maniatis *et al.*, 1982). Even for investigators familiar with the *B. subtilis* system, perhaps the simplest and most direct method for cloning many *B. subtilis* genes involves use of *E. coli* as the initial transformation host. Detection of a specific *B. subtilis* restriction fragment in *E. coli* can be approached by selecting for complementation of an auxotrophic mutation, by colony

hybridization, or by detecting production of a specific protein using antibodies. If a *B. subtilis*/*E. coli* shuttle plasmid is used for cloning *B. subtilis* restriction fragments into *E. coli*, the recombinant molecules can be rapidly returned to *B. subtilis* by transforming competent cells.

7.4. Cloning *B. subtilis* DNA Adjacent to Tn917 Inserts

Tn917 is a transposon that functions in *B. subtilis* to allow transposon mutagenesis (Youngman *et al.*, 1983). Tn917 contains an *erm* gene that specifies erythromycin resistance. In pTV1, Tn917 is present on a small replicon, from pE194, that also contains a *cat* gene (Fig. 4). Since the pE194-derived replicon is temperature-sensitive for replication, the plasmid can be eliminated at high temperature. If selection is maintained for the *erm* gene during temperature elimination of the plasmid, Tn917 transpositions into the chromosome can be detected (see also Section 9). This procedure therefore allows the generation of insertion mutations which are marked by the transposon. In order to clone *B. subtilis* DNA adjacent to Tn917 insertions, Youngman *et al.* (1984) developed two plasmid vectors, pTV20 and pTV21Δ2 (Fig. 5). Each consists of a pBR322 derivative containing a *cat* gene between the *Cla*I and *Bam*HI sites, fused at *Sal*I to pTV5, a pE194-derived replicon containing Tn917. The *Sal*I site in pTV5 is within Tn917. Hence, pBR322-*cat* is inserted within the transposon generating shuttle vectors. This insertion is said to reduce transposition of Tn917 by at least 100-fold in *B. subtilis*. pTV20 and pTV21Δ2 differ in two respects. First, each contains the pBR322-*cat* segment inserted in opposite polarity. Second, neither pTV20 or pTV21 (parent to pTV21Δ2) replicated well in *E. coli*. Therefore, a deletion derivative of pTV21, generated *in vivo*, was isolated, namely pTVB21Δ2, which did replicate in *E. coli*. Although deletion derivatives of pTV20 which replicate in *E. coli* were isolated by *in vitro* removal of sequences which interfere with replication (apparently sequences imme-

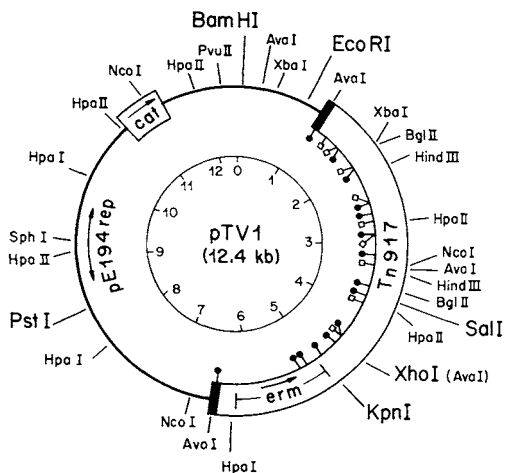


Figure 4. pTV1. This is a temperature-sensitive plasmid vector harboring the transposon Tn917 (Youngman *et al.*, 1983). The complete nucleotide sequence of Tn917 has been determined (Shaw and Clewell, 1985). (↑) = *Mbo*I sites; (∇) = *Taq*I sites; *Mbo*I and *Taq*I sites outside the transposon sequences are not shown.

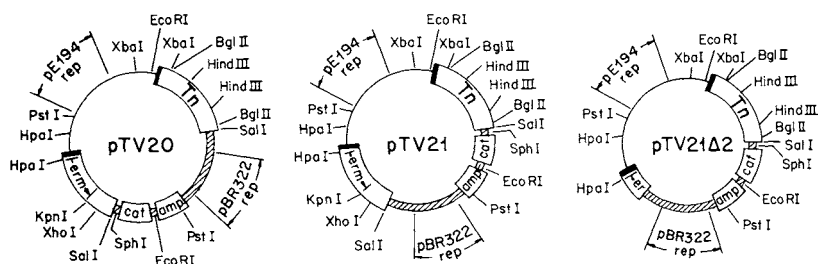


Figure 5. Diagrams of pTV20, pTV21, and pTV21Δ2. Reprinted from Youngman (1987).

diately 3' to *erm*), these deletion derivatives were unnecessary since the method of cloning chromosome fragments into *E. coli* eliminates the interfering sequences.

The use of these plasmids can be illustrated with pTV20 (Fig. 6). Transformation of a linear version of pTV20 (cut at the *erm* distal end of Tn917 with *Xba*I) into a *B. subtilis* strain carrying a single copy of Tn917 in the chromosome can result in a recombinational event between the donor plasmid and the integrated Tn917. This results in the insertion of pBR322 sequences at a site in the chromosome marked by Tn917 (Fig. 6). The desired recombinants are recovered by selecting for cells in which the plasmid *cat* gene is stably maintained, i.e., the cells are chloramphenicol-resistant. If the chromosomal DNA from such recombinants is isolated and then cut with a restriction enzyme for which no sites exist in the transposon arms but one site

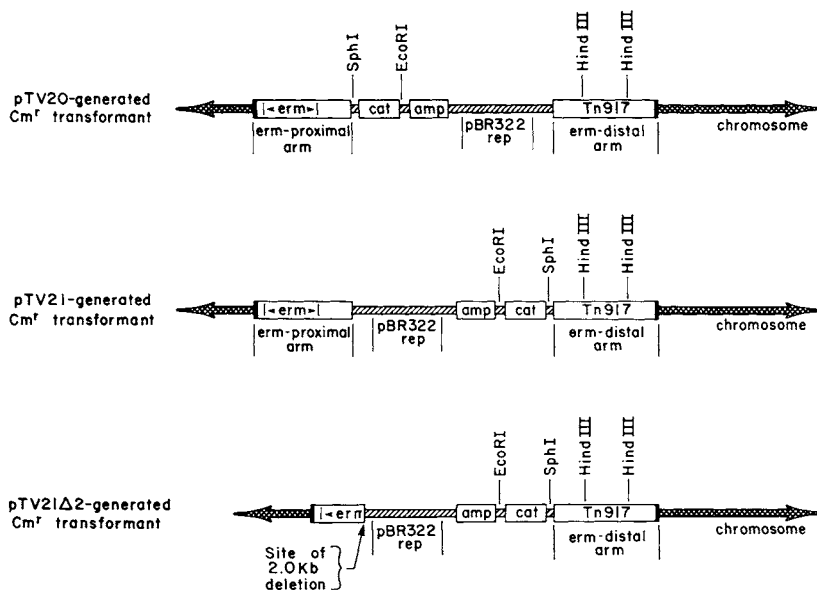


Figure 6. End-products of the recombination between pTV20 and pTV21Δ2 and Tn917 previously transposed into the *B. subtilis* chromosome. Reprinted from Youngman (1987).

exists in the pBR322 sequences (e.g., *EcoRI* or *SphI*) the pBR322 sequences are then placed on a single restriction fragment with chromosome DNA that is to the "right" of the integrated TN917. Since pTV21Δ2 contains pBR322-*cat* in opposite polarity, cutting with *EcoRI* or *SphI* places pBR322 on a restriction fragment that contains chromosome sequences to the "left" of the integrated Tn917. Such restriction fragments can be recovered in *E. coli* after ligation, transformation, and selection for ampicillin resistance.

8. GENE CLONING IN *B. SUBTILIS* BY USE OF BACTERIOPHAGE VECTORS

Two types of gene cloning in *B. subtilis* have been described that involve phage vectors. The first of these uses a clever technique first reported by Kawamura *et al.* (1979). In this procedure, DNA is isolated from mature particles of a *B. subtilis* temperate phage such as ϕ105 or ϕ11 (Iijima *et al.*, 1980). The DNA is digested with a multicutting restriction enzyme (e.g., *EcoRI*) and is mixed with similarly digested *B. subtilis* chromosome DNA. In these studies the *B. subtilis* DNA is frequently obtained by isolating DNA from viruses of an induced defective phage PBSX; PBSX is known to incorporate only fragments of the *B. subtilis* chromosome, and this DNA is somewhat enriched in genetic markers located near the origin of replication of the *B. subtilis* chromosome. After ligation of the phage and chromosome fragments, the mixture is transformed into competent cells of a recombination-proficient strain of *B. subtilis* that is lysogenic for the phage used in the cloning. Transformants containing the desired gene are then selected. For example, if the *trpC* gene were desired, the recipient would be *trpC*-defective, and transformants would be selected on media lacking tryptophan. In this example, two types of recombinational events would lead to the Trp⁺ phenotype. In the more frequent event, the *B. subtilis* chromosome fragment would recombine into the chromosome at its correct location. This is the typical result obtained in transformation of chromosome markers into *B. subtilis*. The minority of Trp⁺ recombinants would result from a recombination event in which the *trpC* fragment flanked by phage DNA fragments inserted itself into the resident prophage. If the inserted DNA does not inactivate functions essential for phage multiplication, a nondefective specialized transducing phage results. However, if the insert disrupts an essential gene, the transducing phage will be defective. This difference reflects the particular phage DNA fragments that flank the cloned gene and the phage being used. For example, certain *HindIII* fragments inserted into ϕ105 can result in a defective, tailless transducing phage (Jenkinson and Mandelstam, 1983). In order to recover specialized transducing particles, the Trp⁺ transformants are induced with mitomycin C, and the phages are used to transduce a *trpC*-defective strain to Trp⁺. Since the phages used in this procedure do not perform generalized transduction, all Trp⁺ cells result from lysogeny by a constructed, specialized transducing phage. This procedure is quite different from plasmid cloning since the introduced gene, e.g., *trpC*, is maintained at one copy per chromosome. Numerous nutritional and sporulation genes have been cloned by this approach (e.g., Hirochika *et al.*, 1981; Ikeuchi *et al.*, 1983; Jenkinson and Mandelstam, 1983).

Cloning by generating specialized transducing phages as described above has changed little since its original description in 1979. Although the technique is clearly useful, modifications in the phage vectors used in cloning would certainly be desirable. Presently, the method does not specifically direct cloned fragments into selected regions of the phage genome. This was presumably useful in the early studies in which the experiments actually located sites in phage genomes where insertions could be made. The result was the identification of sites of insertion which yielded either nondefective or defective phages. Based on the information resulting from such studies it should now be possible to construct bacteriophage mutants that, by analogy with λ , contain "large" nonessential regions containing restriction sites that are not represented elsewhere in the phage genome. Such a phage would allow the insertion of heterologous DNA at the chosen site to yield nondefective phage progeny which are capable of lysogeny. Successful efforts toward this goal have been made. Jenkinson and Deadman (1984) constructed a derivative of $\phi 105$, $\phi 105dCm^r$, that contains a unique *Bgl*II site, and inserts at this site do not inactivate phage replication or lysogeny. The construction of $\phi 105dCm^r$ is described since it depicts an approach that can be used to improve greatly the utility of phage for general cloning in *B. subtilis*. $\phi 105dlys$ is a defective derivative of $\phi 105$ that cannot make tails due to a 3-kilobase deletion. Although the phage is defective, tails can easily be supplied to the $\phi 105dlys$ particles *in vitro* allowing the phage to infect cells. $\phi 105dlys$ DNA contains no *Bgl*II site. Therefore, a small *Hpa*II fragment of pBD64 containing a *cat* gene and a *Bgl*II site (located outside of the *cat* gene) was inserted into the genome of $\phi 105dlys$ yielding $\phi 105dCm^r$, which now is a defective (tailless) specialized transducing phage conferring chloramphenicol resistance on lysogens. A *metC* gene from *B. subtilis* was then cloned into the *Bgl*II site. Although $\phi 105dCm^r$ itself is a useful vector, its construction signaled the development of several other vectors which permit predictable cloning experiments to be performed with temperate phages.

The most sophisticated system for cloning in *B. subtilis* by use of a temperate bacteriophage has recently been developed by J. Errington and colleagues. These investigators constructed nondefective mutants of $\phi 105$ which contain two unique restriction sites, *Bam*HI and *Xba*I, at the location of a 4-kilobase deletion of the $\phi 105$ genome (Errington, 1984; Jones and Errington, 1987). These mutant phages are fully capable of lysogeny in *B. subtilis*, and a valuable alteration introduced into these cloning vectors is the ability to be temperature-inducible. This was achieved by replacing the wild-type phage repressor gene with a mutant allele that renders the repressor protein disfunctional at high temperature. The use of one of these mutant phages for producing gene libraries of *B. subtilis* DNA and recovering specific fragments is elegantly demonstrated in Errington and Jones (1987). The highlights of this cloning approach are as follows: *B. subtilis* DNA is digested with an enzyme which produces cohesive ends that can anneal with *Bam*HI or *Xba*I ends. The digested DNA fragments are size-fractionated and 3- to 4-kilobase fragments are ligated to either *Xba*I- or *Bam*HI-cleaved DNA from phage $\phi 105J27$. The mixture is used to transfect *B. subtilis* protoplasts (at 30°C, since $\phi 105J27$ is thermoinducible). These phages are then used for transduction of appropriate mutants of *B. subtilis* (see Errington and Jones, 1987). By the use of $\phi 105J27$ and similar mutant $\phi 105$ derivatives, it has been possible to clone fragments that complement a variety of mutations in *B. subtilis* including nutritional and sporulation loci. Al

though a single round of cloning does not seem to produce a complete library of fragments, repeats of the cloning procedure have been found to yield recombinant phage that complements all genetic defects tested.

Lastly, a cloning vector has also been constructed using the virulent bacteriophage SPP1. Heilmann and Reeve (1982) inserted a *Bam*HI-sensitive site into a nonessential region of a deletion mutant of SPP1. The resulting phage, designated SPP1V, is infectious for *B. subtilis*, and its DNA is active in transfection. Digestion of SPP1V DNA with *Bam*HI destroys its ability to transfect. *Bgl*II or *Bcl*I fragments inserted into this unique *Bam*HI site render the DNA noncleavable by *Bam*HI. Therefore, small inserts (~4 M Da) in SPP1V DNA can apparently be directly selected (see Behrens *et al.*, 1983). Deletion derivatives of SPP1V have recently been identified, and these presumably will allow cloning of larger DNA fragments (Desmyter *et al.*, 1985).

9. SPECIALIZED CLONING AND EXPRESSION PLASMIDS

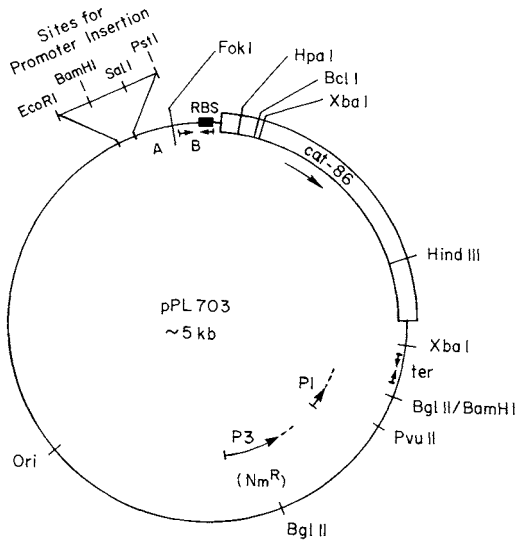
Many highly specialized plasmids have been developed that are useful for a class of experimental manipulation. Below are described specific representatives of three types of vectors.

9.1. Promoter Probe Vectors

DNA in the region 5' to the coding sequence for a gene contains the site to which RNA polymerase binds to initiate transcription. This site, designated the promoter, has been shown to consist of two relevant regions which are named according to their approximate location upstream from the transcription start site. Approximately 35 base pairs upstream from the transcription start site is the "–35" sequence, which is thought to be the initial site of RNA polymerase attachment to DNA. The "–10" regulatory sequence is about 10 base pairs upstream from the start site, and is essential for promoter function. The apparent subsequent binding of RNA polymerase to the –10 sequence may be necessary to "melt" the duplex in preparation for transcription. Much of the regulation of gene expression that is currently known in prokaryotes operates at the level of controlling transcription initiation. This is true in *B. subtilis* and indeed the process of differential gene expression that takes place during sporulation reflects, in large part, changes in RNA polymerase that specifically alter promoter recognition (see Chapter 8, Section 6). The value of understanding promoter function and its regulation has prompted the development of suitable vectors that can be used to clone and/or analyze promoter function *in vivo*.

9.1.1. Vectors for Cloning and Analysis of Promoters

The properties of plasmids that are useful for promoter cloning and analysis can best be described by reviewing the factors that enable one such vector, pPL703, to be used for this purpose (see also Lovett and Mongkolsuk, 1987). The promoter-cloning plasmid pPL703 (~5 kilobase pairs) contains a 1250-base pair segment of *B.*



-144
 EcoRI BamHI SalI PstI
 GAATTCCTCCGATCCGTCGA CCTGCAGSCT TTAACGTAGG CAAAGCTCAG GGTAGACTTT GAATGACAG AACATGACA TATCTCTTGA
 -115
 -85
 -75 FokI -55 AAGGATGATT GGGTGGTGA AAACAGATAA AATCTCCCTCC TGAATACAGT AATACACATT CAGGAGGAGA TAAAAA TTG TTT AAA CAA
 -25 -1 +1 Aha III
 MET Phe Lys Gln

ATA GAC GAA AAT TAT CTG CGA AAA GAG CAC TTT CAC CAT TAT ATG ACG TTA ACC CGA TGC TCA TAT AGC TTG GTG
 Ile Asp Glu Asn Tyr Leu Arg Lys Glu His Phe His His Tyr MET Thr Leu Thr Arg Cys Ser Tyr Ser Leu Val
 Bcl I Xba I
 ATC AAT CTA GAC ATC ACG AAA TTG CAT GCA ATA TTA AAA GAA AAA AAG CTG AAA GTA TAT CCT GTG CAA ATT TAT
 Ile Asn Leu Asp Ile Thr Lys Leu His Ala Ile Leu Lys Glu Lys Lys Lys Val Tyr Pro Val Gln Ile Tyr
 TTG TTA GCA AGA GCT GTG CAA AAA ATT CCT GAG TTT CGG ATG GAT CAA GTG AAC GAT GAA CTT GGT TAC TGG GAG
 Leu Leu Ala Arg Ala Val Gln Lys Ile Pro Glu Phe Arg MET Asp Gln Val Asn Asp Glu Leu Gly Tyr Trp Glu
 ATT CTC CAT CCT AGT TAT ACG ATT CTA AAT AAA GAA ACA AAG ACG TTT TCA AGT ATT TGG ACG CCT TTT GAT GAA
 Ile Leu His Pro Ser Tyr Thr Ile Leu Asn Lys Glu Thr Lys Thr Phe Ser Ile Trp Thr Pro Phe Asp Glu
 AAC TTT GCT CAG TTT TAT AAA AGC TGT GTA GCC GAT ATT GAA ACA TTT AGC AAA AGC AGC CTA TTT CCG AAA
 Asn Phe Ala Gln Phe Tyr Lys Ser Cys Val Ala Asp Ile Glu Thr Phe Ser Lys Ser Asn Leu Phe Pro Lys
 CCT CAT ATG CCA GAA AAC ATG TTC AAT ATT TCA AGT CTA CCG TGG ATT GAT TTT ACT TCT TTT AAC CTT AAT GTA
 Pro His MET Pro Glu Asn MET Phe Asn Ile Ser Ser Leu Pro Trp Ile Asp Phe Thr Ser Phe Asn Leu Asn Val
 TCT ACA GAT GAA GCT TAT TTA CTG CCT ATA TTT ACG ATA GGC AAA TTT AAG GTG GAA GGA AAA ATC ATT TTG
 Ser Thr Asp Glu Ala Tyr Leu Leu Pro Ile Phe Thr Ile Gly Lys Phe Lys Val Glu Glu Gly Lys Ile Ile Leu
 CCG GTT GCC ATA CAA GTA CAT CAT GCT GTT TGT SAT GGC TAT CAT GCC GGT CAA TAT GTT GAA TAT TTG AGG TGG
 Pro Val Ala Ile Gln Val His His Ala Val Cys Asp Gly Tyr His Ala Gly Gln Tyr Val Glu Tyr Leu Arg Trp
 CTT ATT GAA CAT TGT GAC GAG TGG TTA AAT SAT TCA TTG CAT ATT ACC TGAAGACAAAAGGTGCTCCATGAAAATCTAGAA
 Leu Ile Glu His Cys Asp Glu Trp Leu Asn Asp Ser Leu His Ile Thr *

695 745
 GATGAAGGCA GAGTGAATG AGCTGGCTT TTGTTTGT AATGATAATG TGCCAAAGGT TTAATGATG AACAATGTT CTTTTCGGC
 795 845
 TTCTTTTTCG GTGCTTTGTA AATCTTATAT GTGATAATTA ATGTAATATA AGGGAATTA GTGGAAGGCG AATAAGGAT GGAGAAGTC
 895 945
 TTCTTCACA TGTAGGTGTG AAAATAAATG AATGATATAG AATGATTCGG CAATTCAGTG TACCCGAGCG AGAGATTITG AAGAGTCGAG
 995 1045
 TAGACAAGA AATTGAACGA ATGGAAGAG ATCAAGATTT ACTCATTTAT TATCAATTGA TGTGCTTTCG ACATCAATAT ATGTATAGATT
 1095 Bgl II
 ACATTAAAC TGTAGACTTT AATAAAAATA GACCAACCAT TTATCCCTC TTATGATCT

pumilus DNA that contains a promoter-deficient version of *cat-86* inserted between the *Eco*RI and *Bam*HI sites of pUB110 (Fig. 7; Mongkolsuk *et al.*, 1983). The *cat-86* gene is oriented opposite to vector transcripts and is only expressed in *B. subtilis* when a promoter is inserted into any of four unique restriction sites (*Eco*RI, *Bam*HI, *Sal*I, *Pst*I) located 144 base pairs upstream from the *cat-86* coding sequence. *cat-86* specifies chloramphenicol acetyltransferase and therefore activation of *cat-86* by promoter insertion enables the derivative plasmid to confer chloramphenicol resistance on *B. subtilis*. The region of pPL703 derived from pUB110 (~3.8 kilobase pairs) provides an origin of replication and a neomycin/kanamycin resistance gene whose expression is unrelated to, and unaffected by, promoters cloned upstream from *cat-86*. pPL703 and many promoter-containing derivatives are stably maintained in *B. subtilis* at high copy number in the absence of selection for the plasmid. Four properties of pPL703 enable it to be used effectively for promoter cloning. First, expression of *cat-86* resulting from promoter insertion allows pPL703 to confer high-level chloramphenicol resistance to *B. subtilis*; this provides a strong positive selection for plasmids that have acquired a promoter. Furthermore, spontaneous chloramphenicol-resistant variants of *B. subtilis* resulting from a chromosome mutation have never been detected in our cloning experiments. Second, the 144-base pair region between the site of promoter insertion and the *cat-86* gene contains 12 translation termination codons with at least two in each reading frame (Duvall *et al.*, 1983). Thus, if translation is initiated in a cloned promoter, it is blocked prior to entering *cat-86*. Third, the region 3' to *cat-86* contains a transcription termination site that is more than 90% efficient in both orientations (Mongkolsuk *et al.*, 1985). Thus, transcripts initiated in cloned promoters do not enter the vector, and vector transcripts do not enter *cat-86*. Fourth, chloramphenicol acetyltransferase activity can be quantitatively measured by a simple colorimetric procedure and by a highly specific and sensitive assay that directly measures acetylation of radioactive chloramphenicol (Shaw, 1983). pPL703 can replicate only in *B. subtilis*, although a derivative, pPL703BC, has been developed which replicates in both *B. subtilis* and *E. coli*. In Table III are shown the properties of pPL703 and representative promoter-cloning plasmids that also use activation of a selectable phenotype as an indicator of promoter cloning (see also Lovett and Mongkolsuk, 1987).

In addition to plasmids which use activation of a selectable phenotype to detect promoter insertion, two plasmids use activation of a scorable, not a selectable, phenotype. Plasmid pCED6 (Donnelly and Sonenshein, 1984) contains a promoterless *lacZ* gene downstream from a unique *Hind*III site, and promoter activation of *lacZ* results in a plasmid that confers on *B. subtilis* cells the ability to produce β -galactosidase. Therefore, transformant colonies that have acquired a promoter-containing version of pCED6 are blue on media containing the indicator dye X-gal

Figure 7. Promoter-cloning plasmid pPL703. pPL703 consists of a 1250-base pair segment of *B. pumilus* DNA inserted between the *Eco*RI and *Bam*HI sites of pUB110 (Mongkolsuk *et al.*, 1983). This inserted DNA contains a promoterless gene, *cat-86*, preceded by a multicloning site linker for promoter insertion. The *cat-86* gene is followed by a strong transcription termination signal (*ter*; Mongkolsuk *et al.*, 1985). The sequence of the 1250-base pair fragment is shown, as is the deduced translation product of *cat-86*.

Table III. Promoter Probe Plasmids for *B. subtilis*^a

Feature	Selectable phenotype			Scorable phenotype	
	pPL703	pCPP-3	pGR72 pGR73	pTG402	pCED6
Approximate size (kilo- base point)	5	5.6	8.4	9	12.5
Copy number ^b Host	High <i>B. subtilis</i>	High <i>B. subtilis</i> , <i>E. coli</i>	High <i>B. subtilis</i> , <i>E. coli</i>	High <i>B. subtilis</i> , <i>E. coli</i>	High <i>B. subtilis</i> , <i>E. coli</i>
Neutral plasmid marker ^c	Neo [®] /Kan [®]	Neo [®] /Kan [®]	Neo [®] /Kan [®]	Cm [®]	Neo [®] /Kan [®] (Amp [®] in <i>E. coli</i>)
Promoter-indicator gene	<i>cat-86</i> (from <i>B. pumilus</i>) Cm [®]	<i>cat</i> (from pC194)	<i>cat</i> (from Tn9)	<i>xyIE</i> (from TOL plasmid)	<i>lacZ</i> (from <i>E. coli</i>)
Host phenotype when indicator gene is ex- pressed		Cm [®]	Cm [®]	Yellow colonies (sprayed with catechol)	Blue colonies (on X-gal)
Unique sites for promo- ter cloning	<i>EcoRI</i> , <i>BamHI</i> , <i>SalI</i> , <i>PstI</i>	<i>EcoRI</i> (<i>BamHI</i> in pCPP-4)	<i>HindIII</i>	<i>BamHI</i>	<i>HindIII</i>
Reference	Mongkolsuk <i>et al.</i> , 1983	Band <i>et al.</i> , 1983	Lin <i>et al.</i> , 1985	Zukowski <i>et al.</i> , 1983	Donnelly and Sennsheim, 1984

^aData from Lovett and Mongkolsuk.

^bThe copy number of pPL703 is about 30. The copy numbers of the other plasmids have not been reported, but each replicates in *B. subtilis* using a replicon derived from a high-copy *S. aureus* plasmid, either pUB110 or pC194 (Ehrlich, 1977; Cryczan *et al.*, 1980b).

^cResistance to neomycin (Neo[®]) and kanamycin (Kan[®]) are specified by the same gene. Cm[®] and Amp[®] designate resistance to chloramphenicol and ampicillin.

(5-bromo-4-chlor-3-indolyl- β -D-galactopyranoside). The plasmid pTG402 contains the promoter-indicator gene *xylE* (Zukowski *et al.*, 1983). Activated forms of this gene confer on *B. subtilis* cells a yellow colony color when the colonies are sprayed with catechol.

9.1.2. Transposition of a Promoterless Gene

Certain promoters can be regulated by host effector molecules. If promoters of this type are cloned onto multicopy vectors, such as shown in Table I, the amplified number of promoters can, in theory, titrate the effector or regulatory molecules and the regulation may be lost. An example of this was recently shown (Osborne *et al.*, 1985). In addition, promoters that control many sporulation genes may block host sporulation when they are placed on multicopy plasmids. Hence a need has existed for a promoter analysis vector that does not elevate the copy number of the promoter under study. Several such vectors were recently constructed by Youngman and colleagues by use of the transposon Tn917.

Variations of pTV1 (Fig. 4) have been constructed in which Tn917 contains promoterless genes in addition to the *erm* gene (Youngman *et al.*, 1985). A site located between the left inverted repeat of Tn917 and the *erm* gene has been identified into which inserts of promoter-deficient genes such as *lacZ* or *cat-86* have been made (Fig. 8). Indeed one vector, pTV53, contains both *lacZ* and *cat-86* tandemly inserted into Tn917.

The use of the promoter-probe derivatives of Tn917 is similar to that seen with Mudlac phages in *E. coli*. An illustration of the use of these plasmids can be made using pTV51. pTV51 consists of a temperature-sensitive replicon which carries a *tet* gene (tetracycline resistance) and a version of Tn917 containing a promoterless *cat-86*. Introduction of pTV51 into *B. subtilis* results in stable inheritance of tetracycline and erythromycin resistance at 32°C. Since *cat-86* lacks a promoter, the

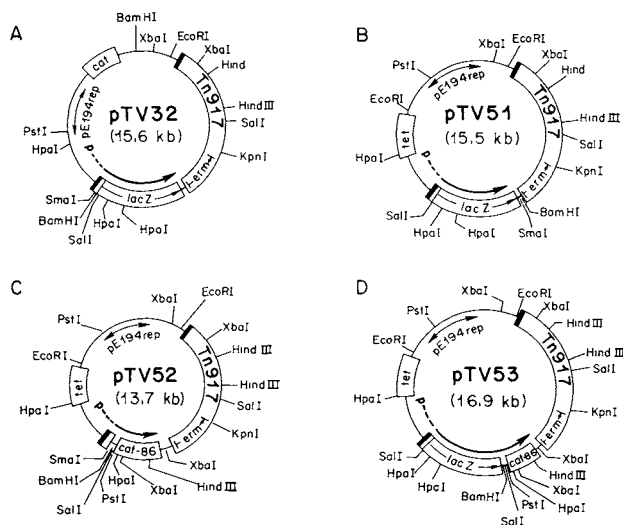


Figure 8. Derivatives of Tn917 containing promoterless *cat-86* and/or *lacZ* genes.

host cells remain sensitive to chloramphenicol at 10 µg/ml. Growth of such cells at 47°C under conditions that select for maintenance of *erm* (1 µg/ml of erythromycin and 25 µg of lincomycin) selects for cells in which Tn917 has become inserted into the *B. subtilis* chromosome. Since the elevated temperature blocks replication of pTV51, the cells are tetracycline-sensitive. The transposition event places both *erm* and *cat-86* into various chromosomal sites. Inserts that place *cat-86* under control of a chromosomal promoter result in a chloramphenicol resistance phenotype.

pTV53 contains promoterless *cat-86* and *lacZ* genes inserted into Tn917 in tandem. This plasmid was designed to permit rapid detection of chromosomal promoters which are active only in the postexponential growth phase. Promoters of this type include those governing transcription of spore genes. Transposition of Tn 917 carrying the *lacZ* and *cat-86* genes results in the chloramphenicol resistance phenotype (and β-galactosidase activity) only if the transposon places the two genes under control of a promoter that functions during exponential growth. When the two promoterless genes are placed under control of a promoter that is active only during postexponential growth, the cells become β-galactosidase-positive but remain sensitive to chloramphenicol. The different chloramphenicol resistance phenotypes which result when Tn917 inserts downstream from the two classes of promoters is due to the fact that expression of chloramphenicol resistance requires activation of *cat-86* during the active growth phase of the cells.

9.2. Expression Vectors

Genes from gram-positive bacteria seem to express well, as a rule, when cloned into *B. subtilis*. This suggests that the regulatory sequences which are associated with "gram-positive" genes function in *B. subtilis* to signal the initiation of transcription and translation. Cloned gram-positive genes that lack a promoter can probably be expressed if they are cloned into plasmids at sites that are actively transcribed from promoters located in the vector. For example, *cat-86* deleted of its natural promoter is expressed when the gene is inserted into the *Eco*RI site of pUB110 in a counterclockwise orientation (Williams *et al.*, 1981a). This location in pUB110 is under active counterclockwise transcription due to a promoter located near the *Pvu*II site on the pUB110 map. By contrast, genes from a typical gram-negative bacterium, *E. coli*, are either poorly expressed in *B. subtilis* or not detectably expressed in *B. subtilis* (e.g., Goldfarb *et al.*, 1981). This is apparently due to the lack of utilization by *B. subtilis* of the natural sequences associated with *E. coli* genes that signal transcription and translation initiation (McLaughlin *et al.*, 1981; Moran *et al.*, 1982). Similarly, cDNA corresponding to a typical eukaryotic gene is also poorly expressed in *B. subtilis* unless extensive manipulations are made to provide the gene with appropriate promoter and ribosome binding site sequences. Eukaryotic genes and *E. coli* genes, which have been expressed in *B. subtilis* by providing transcription and/or translation initiation signals, can produce catalytically active proteins. Hence, the genetic code used in *B. subtilis* is probably identical to that used in *E. coli* and eukaryotes.

The simplest way to express a gene from *E. coli* or from a eukaryotic in *B. subtilis* is to fuse the gene in-frame to a gene that is known to be expressed in this organism. For example, the *E. coli trp* promoter is virtually nonfunctional in *B. subtilis* (S. Mongkolsuk, unpublished), and the ribosome binding sites associated

with the *trp* operon are probably too weak to permit translation initiation. However, a region of the *E. coli trp* operon spanning a portion of *trpD* and all of *trpC* can be efficiently expressed when fused to *cat-86* at the *HindIII* site (Williams *et al.*, 1981b; Harwood *et al.*, 1983). Similarly, the major antigen of foot-and-mouth disease virus was expressed in *B. subtilis* by fusing the viral gene in-frame with an *erm* gene (Hardy *et al.*, 1981). Such in-frame fusions require knowledge of the nucleotide sequence of both the foreign gene and the gene in to which the insertion is to be made.

Since the blocks to heterologous gene expression are at the steps of transcription and translation initiation, expression plasmids designed to allow synthesis of nonfused foreign proteins should provide a promoter followed by a strong ribosome binding site, AGGAGG, that precedes the ATG codon of the foreign gene by 5–10 base pairs. Williams *et al.* (1981b) constructed a versatile expression vector capable of expressing mouse dihydrofolate reductase, the *E. coli recA* gene (deVos *et al.*, 1983), as well as other genes of eukaryotic and prokaryotic origin. Similar efforts by other investigators have allowed the expression of many *E. coli* genes in *B. subtilis*.

The most sophisticated expression plasmid yet reported for *B. subtilis*, pLIQ-1 was developed by Yansura and Henner (1984) and permits IPTG-inducible expression of foreign genes. The vector portion of pLIQ-1 consists of pUB110 with the pBR322 replication origin inserted at the *BamHI* site. In addition, the plasmid contains the *lacI* gene (encoding the *lac* repressor) under control of the *B. licheniformis* penicillinase promoter and ribosome binding site. At a second location on pLIQ-1 was inserted a strong promoter with the *lac* operator sequences 3' to the –10 regulatory region. This was followed by a strong ribosome binding site and the human gene which specifies leukocyte interferon A. The construction permits IPTG (an inducer of the *E. coli lac* operon) to induce interferon in *B. subtilis*. A two-plasmid expression system developed by Le Grice *et al.* (Le Grice *et al.*, 1987) is in principle very similar to pLIQ-1. pLIQ-1 is a good example of the principles that have been found necessary to achieve expression of prokaryotic and eukaryotic genes in *B. subtilis*. However, it is likely that no single expression plasmid is going to be ideal for obtaining maximum expression of all foreign genes.

9.3. Secretion Vectors

Expression plasmids such as pLIQ-1 (see Section 9.2) permit the accumulation of a foreign gene product within *B. subtilis*. This may not be the ideal type of vector for producing extremely high quantities of gene product for two reasons. First, very high levels of a gene product which are maintained within *B. subtilis* may be toxic to the cell or at least reduce the level of gene expression. Second, in *E. coli* it has been found that very high production of a gene product that is maintained intracellularly can cause accumulation of the protein as a crystalline-like structure generally visible at the cell poles. This structure consists of the foreign protein in a nonnative state which must be renatured to obtain the biological activity. The efficiency of the renaturation can be quite low in some cases. Thus, it is possible to produce high yields of protein product but to have low yields of biological activity.

To avoid the problems noted above, efforts have been made to develop secretion vectors, in which the foreign gene product is expelled from the producing cell

concomitant with synthesis (see Chapter 9). *Bacillus subtilis* has been viewed as an ideal host for development of secretion vector systems since many proteins in this organism are naturally secreted at a high level. The general approach that has been followed requires cloning a gene whose product is secreted by *B. subtilis*. Genes in this class include those encoding α -amylase and proteases. The region of a gene encoding the signal peptide can be determined by sequencing the mature extracellular protein and sequencing the gene. This reveals which amino acids are cleaved from the precursor protein during secretion. The DNA encoding the signal peptide is then fused in-frame to a foreign gene such as that encoding human interferon. The result should be intracellular synthesis of interferon with a signal peptide attached to the amino terminus and cleavage of the signal peptide during secretion. This type of vector was first developed by Palva and co-workers (Palva, 1982; Palva *et al.*, 1982, 1983) and may be useful for high-level production of foreign proteins. One problem that may be encountered is that secretion places a foreign protein into extracellular medium containing high quantities of proteases which are also secreted by the bacterium. Protease-deficient mutants such as recently reported may therefore greatly aid the use of secretion vectors in this system (Wells *et al.*, 1983; Yang *et al.*, 1984; Kawamura and Doi, 1984).

10. RECOMBINATION BETWEEN CLONED SEGMENTS OF *B. SUBTILIS* DNA AND THE *B. SUBTILIS* CHROMOSOME

Cloned regions of the *B. subtilis* chromosome can recombine with the host chromosome. The site of recombination is determined by the region of homology shared by the cloned fragment and the corresponding region on the *B. subtilis* chromosome (Haldenwang *et al.*, 1980). This principle allows cloned fragments of the *B. subtilis* chromosome to direct recombinational events that can have two effects: the insertion of foreign sequences into the *B. subtilis* chromosome and the generation of deletions within the chromosome (Rosenbluh *et al.*, 1981; see Chapter 4, Section 3.2).

11. GENE BANKS

Fragments of the *B. subtilis* chromosome have been cloned into *E. coli* using either phage or plasmid vectors (Rapaport *et al.*, 1979; Ferrari *et al.*, 1981). The resulting gene banks or libraries likely contain all or nearly all of the *B. subtilis* chromosome in overlapping fragments. Three approaches exist to identifying specific regions of the *B. subtilis* chromosome in these gene banks: First, if the cloned DNA expresses in *E. coli*, one can select for recombinant plasmids that complement specific mutations in *E. coli*. Second, one can transform *B. subtilis* with the clones in order to repair to wild-type selected chromosomal mutations. Last, colony or plaque hybridization techniques can also be used to identify specific sequences. For example, ribosomal RNA from *B. subtilis* can be used as a probe to detect the corresponding gene in λ or plasmid vectors.

12. FUTURE PROSPECTS FOR GENETIC MANIPULATION IN *BACILLUS SUBTILIS*

Continued studies of the molecular genetics of *B. subtilis* will undoubtedly provide insight in the regulatory mechanisms governing both DNA replication and gene expression, as is evidenced by other chapters in this volume. However, the technical area in the genetic manipulation of *B. subtilis* which remains in need of much improvement is gene cloning.

Cloning in *B. subtilis* is presently performed by several variations which use either phage or plasmids as vectors and competent cells or protoplasts as the transformation recipient. Among the methods available for cloning, the system of Kawamura *et al.* (1979), which permits the *in vitro* construction of specialized transducing phages, has the greatest potential for use as a general-purpose, primary cloning system in *B. subtilis*. This system seems extremely useful since it can be applied to both homologous and heterologous gene cloning. Furthermore, the copy number of the inserted gene is low (one or two) and recovery of the cloned gene appears relatively simple. The phage system is most useful in isolating genes for which a selectable phenotype exists. However, alternative approaches for identifying cloned heterologous genes exist such as plaque or colony hybridization. Thus, the system can probably be used to identify genes based on criteria other than expression. For example, perhaps temperate phage, such as $\phi 105$, derivatives can be constructed which have a gene such as *lacZ* inserted into a nonessential region of the genome. Lysogens would express β -galactosidase and therefore exhibit blue colonies on media containing X-gal. Perhaps this addition to the Errington system would enable the identification of cloned DNA fragments without requiring a positive selection. Other approaches certainly exist which would make the phage-cloning system more versatile and perhaps simpler to use than the available approaches.

A second technical area that requires improvement is the use of *B. subtilis* for secretion of the products of cloned genes. Secretion vectors have been developed (e.g., Palva *et al.*, 1983); however, problems have been encountered with the stability of the secreted products of certain foreign genes. It remains unclear whether this instability is in certain cases due only to protein degradation in the extracellular state or whether certain foreign proteins become trapped in the membrane and are consequently degraded. These are important problems to resolve if *B. subtilis* is to have broad applications in industry for the production of foreign gene products.

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Plasmid Replication and Stability

7

MARILYN E. NUGENT

1. INTRODUCTION

The maintenance of bacterial plasmids is dependent on their faithful replication and on their accurate partitioning at cell division. Under defined growth conditions, specific plasmid types are maintained at a characteristic number of copies per chromosome. Copy number is controlled by the machinery which regulates plasmid replication. Although there are differences in the nature of replication control mechanisms, certain features are common to most plasmids that have been investigated. For example, negatively acting control elements have been identified in many systems; in a number of cases RNA replication primers and overlapping countertranscripts appear to be the controlling elements. In other cases plasmid-encoded proteins are positively required for replication.

Most of the early vectors used for cloning in *Bacillus subtilis* were plasmids derived from antibiotic-resistant strains of *Staphylococcus aureus*. The replication mechanisms of these vectors have been more intensively studied than those of naturally occurring *Bacillus* plasmids. Recently, however, antibiotic resistance plasmids from *Bacillus* strains have been identified and cryptic plasmids have been labelled with selectable markers. The replicons of these plasmids are being used increasingly as the basis of a new generation of *Bacillus* vectors in which earlier problems of plasmid instability have been largely overcome.

2. REPLICATION MECHANISMS OF PLASMIDS ISOLATED FROM *BACILLUS* STRAINS

2.1. pTB19

pTB19, a 26.5-kilobase pair kanamycin and tetracycline resistance plasmid, was isolated from a thermophilic *Bacillus* (Imanaka *et al.*, 1981). This plasmid contains two replication determinants, *repA* and *repB* (Imanaka *et al.*, 1984). Deletion deriva-

tives of pTB19 containing *repA* can replicate only in *B. subtilis* whereas derivatives containing *repB* can replicate in *B. subtilis* and *B. stearothermophilus*.

pRATII is a 2.6-kilobase pair plasmid containing *repA* from pTB19 and the kanamycin resistance gene from pUB110 (see Section 3.1). This plasmid has been completely sequenced (Imanaka *et al.*, 1986) and a large open reading frame (ORF) encoding a 396-amino acid protein was found in the *repA* portion of the plasmid. The *repA* gene product is required for plasmid replication and is active *in trans*. The region where RepA acts has been narrowed down to a 374-base pair fragment which contains 3.5 direct repeats of a 24-base pair sequence followed by a region rich in A+T. Immediately upstream of these direct repeats is the sequence TTATCCACA. In *E. coli* this sequence is recognized by the DNaA replication protein and serves as the replication origin for plasmids R1, R100, P1, F, and CloDF13 (Fuller *et al.*, 1984). pRATII is a low-copy-number plasmid existing at approximately eight copies per chromosome. For several low-copy-number *E. coli* plasmids, such as F, P1, R6K, and pSC101, a plasmid-encoded protein regulates replication by binding to directly repeated sequences and by analogy a similar mechanism may also control the replication of pRATII.

Replication control by the *repB* region of pTB19 has been studied using the deletion derivative pRBHI (Ano *et al.*, 1986). The replication origin region of pRBHI contains a 63-base pair inverted repeat upstream of the *repB* coding region. pRBHI normally has a copy number of 40 copies per chromosome; however, mutants have been isolated that have a fivefold increase in copy number. These mutants are altered in the *repB* coding region, resulting in the replacement of a glycine residue by an acidic amino acid (Ano *et al.*, 1986). The large inverted repeat upstream of *repB* is surrounded by two promoters, P1 and P3, transcription from which produces overlapping countertranscripts (Fig. 1). The transcript from P1, RNA1, may serve as the primer of DNA replication. Transcription from this promoter was repressed *in vivo* by a trans-acting plasmid gene product. It has been proposed that the *repB* product acts as the repressor in a replication control system that is analogous in some ways to that of the *E. coli* plasmid ColE1. Replication of ColE1 is negatively controlled by a transcript, RNAI, and a 63-amino acid polypeptide, Rom, which enhances the binding of RNAI to the replication preprimer RNAII (Som and Tomizawa, 1983). The pRBHI transcript RNA3 may act as an inhibitor analogous to RNAI of ColE1. RepB may have a function similar to that of Rom, enhancing the binding of RNA3 to RNA1. Synthesis of RepB could be regulated by the small transcript RNA4, which may mask the ribosomal binding sequence of RNA2.

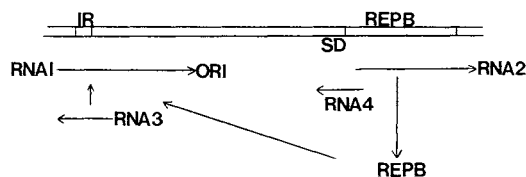


Figure 1. Model for replication control in pRBHI (model proposed by Ano *et al.*, 1986). The transcript from P1, RNA1, may serve as a primer for replication. Primer activity may be regulated by RepB enhancing the binding of RNA3 to RNA1 and hence resulting in inhibition of primer activity. Synthesis of RepB may be regulated by RNA4.

2.2. pIM13

pIM13 is a 2.2 kilobase pair plasmid from *B. subtilis* that confers resistance to macrolide-lincosamide-streptogramin B antibiotics (MLS) (Mahler and Halvorson, 1980). It has a copy number of approximately 200 per cell in *B. subtilis* and 30 in *S. aureus*. Replication and maintenance functions are contained within a 865-base pair *CfoI* to *HindIII* fragment (Monod *et al.*, 1986). This region contains an open reading frame (ORF3) encoding a 16-KDa protein which is likely to be essential for replication. Sequence analysis of pIM13 indicates a strong similarity to the *S. aureus* plasmid pE5 (Monod *et al.*, 1986). However, pE5 has a copy number of just 4–5 in *B. subtilis*. This difference may be explained by a 100-base pair sequence present in ORF3 of pE5 but absent in pIM13. pIM13 is a potentially useful cloning vector for *B. subtilis* as it is stably maintained and appears to tolerate the acquisition of at least several kilobases of foreign DNA without a reduction in copy number.

2.3. pBAA1

pBAA1 is a cryptic plasmid isolated from an industrial strain of *Bacillus* (Devine *et al.*, 1987). It is 6.8 kilobase pairs in size, has a copy number of approximately six per chromosome, and is segregationally stable under industrial fermentation conditions. A 2.2-kilobase pair fragment of pBAA1 replicated with a copy number and a segregational stability similar to that of the parental plasmid. Subcloning and *BAL* 31 deletions of this fragment delineated the basic replicon to a 1.4-kilobase pair fragment. *BAL*31 deletions localized two regions of this fragment that are essential for replication in *B. subtilis*. One region contained an open reading frame encoding a protein with a molecular mass of approximately 34,000. This protein has homology with the gene A protein of pC194 and the α protein of pUB110. The second region contains a series of inverted repeats and a 17-base pair sequence homologous to the replication origins of pC194 and pUB110. It is likely that these three plasmids have similar mechanisms of replication.

3. VECTORS DERIVED FROM *S. AUREUS* STRAINS

More information is available on plasmids derived from antibiotic-resistant strains of *S. aureus* such as pC194, pE194, pT181, and pUB110 (see Chapter 6), which can replicate in certain strains of *Bacillus*.

3.1. pUB110

pUB110 was isolated from a kanamycin-resistant strain of *S. aureus* (see Chapter 6, Section 3.1) and can be maintained in *B. subtilis*, *B. stearothermophilus*, *B. licheniformis*, *B. megaterium*, and *B. pumilis*. In *B. subtilis*, pUB110 has a copy number of 30–50 copies per chromosome with a majority of the plasmid molecules (60–80%) bound to the cell membrane. Two types of membrane binding have been described. Type I binding requires the product of one of the host initiation genes, *dnaB1*, and is high-salt-resistant (Winston and Sueoka, 1980). Type I binding is necessary for *in vivo* replication but the membrane binding site has not been identi-

fied. Tanaka and Sueoka (1983) described four separate binding areas of pUB110 which were able to bind *B. subtilis* membranes *in vitro*—a binding reaction designated type II. This binding has no requirement for the product of *dnaB1* and is salt-sensitive. Two of the four type II binding areas flank the origin of replication (BA1 and BA2) whereas the remaining two (BA3 and BA4) are further away. Sueoka *et al.* (1984) proposed that type II binding is necessary for initial binding to a specific site or sites on the membrane. Subsequently, the type I membrane complex, the putative initiation and replication complex, may be built up around the replication origin.

In addition to the kanamycin resistance gene, sequence data for pUB110 indicates two sizable open reading frames, ORF α and ORF β , and two small ORFs, δ and γ (McKenzie *et al.*, 1986). All of these reading frames are in the same orientation which coincides with the direction of replication. ORF α corresponds to a 334-amino acid protein which may be essential for the initiation of replication. The putative protein is highly basic and contains about five short hydrophobic regions. Of particular interest is a consensus amino acid sequence also found in the DNA binding regions of the phage λ and *lac* repressors. The 334-amino acid protein may bind to a specific DNA sequence of pUB110 and also to the membrane.

The sequence around the pUB110 origin of replication shows none of the long, inverted, complementary repeat sequences which have been reported around the replication origins of two other *S. aureus* plasmids, namely, pC194 and pE194. Plasmid pBC16, which replicates in *B. cereus* and *B. subtilis*, has extensive homology with pUB110 (Polak and Novick, 1982). This homology includes the region of the replication origin and the membrane binding areas BA1, 2, and 4. The BA3 region of pUB110 is not present in pBC16.

3.2. pE194

pE194 was originally isolated from *S. aureus* by Iordanescu (1976) (see Chapter 6, Section 3.2.). It carries the *ermC* determinant which confers resistance to macrolide-lincosamide-streptogramin (MLS) antibiotics. Replication of pE194 is temperature-sensitive and it does not replicate above 45°C in *B. subtilis*. A region sufficient to drive autonomous replication is contained within a 0.9-kilobase fragment (Gryczan *et al.*, 1982). This segment contains the replication origin and specifies a *trans*-acting function (*rep*) required for stable replication and a negatively acting copy control function which is the product of the *cop* gene. The target sites for the *rep* and *cop* gene products are within this region. Two major transcripts are found within the replication region which may be similar to the primer and regulatory elements of ColE1. The superabundance of these transcripts in the Cop6 mutation may reflect the direct effect of this mutation on transcription or possibly the inactivation of the repressor. The Cop repressor maintains a copy number of 10 copies per chromosome at 30°C. Mutants with copy numbers ranging from 25 to 200 copies per chromosome have been isolated (Weisblum *et al.*, 1979).

3.3. PT181

The *S. aureus* plasmid pT181 is similar to many other tetracycline-resistant staphylococcal plasmids, e.g., pT127 and pSN1 (Iordanescu, 1976). These plasmids

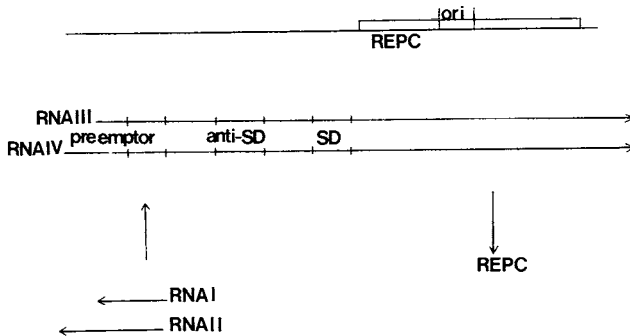


Figure 2. Replication control of pT181. RepC is translated from the transcripts RNAIII and RNAIV. The ribosomal binding sites of these transcripts can potentially base-pair with a region called anti-SD. This base pairing is prevented by a preemptor region of the transcript which can base-pair with the anti-SD region and leave the SD region accessible for ribosome binding. The countertranscripts RNAI and RNAII may interact with the preemptor region and block its base-pairing with anti-SD resulting in an overall inhibition of RepC synthesis.

are approximately 4.5 kilobase pairs in size, encode inducible resistance to tetracycline, and have a copy number of around 20 copies per cell (Iordanescu *et al.*, 1978). pT181 has been fully sequenced and contains four open reading frames for polypeptides of more than 50 amino acids. The largest product, with a molecular mass of 37,500, is the RepC protein. This protein is required for replication, is *trans*-active, and is rate limiting (Manch-Citron *et al.*, 1986). The replication origin is contained within the *repC* coding region. Two short transcripts, RNAI and RNAII, regulate replication negatively and some pT181 copy mutants produce reduced amounts of these transcripts. RNAI and II are transcribed from the region specifying the 5' end of the untranslated *repC* leader but in the opposite direction to *repC*. These countertranscripts are thought to act by interfering with *repC* translation. A model proposed by Novick *et al.* (1985) based on possible interactions between computer-predicted secondary structures, suggests that the interaction of RNAI and II with the *repC* leader alters the secondary folding of the mRNA such that the *repC* ribosome binding site region is unavailable for translation (Fig. 2). A preemptor sequence keeps the ribosome binding site in an accessible conformation. The use of inhibitory countertranscripts is also found in ColE1 and IncFII plasmids.

Two newly discovered *cis*-specific elements are important for normal plasmid function. One is *palA*, a palindromic sequence required for lagging strand synthesis (Gruss *et al.*, 1987). Plasmids with *palA* deletions have very low copy numbers and are unstable. Deletions in the other element, *cmp* (competition), result in a reduced ability to compete with other coresident pT181 plasmids for RepC (Gennaro and Novick, 1986).

3.4. pC194

Plasmid pC194 (see Chapter 6, Section 3.3) is one of several small plasmids discovered in *S. aureus* reported by Iordanescu *et al.* (1978). This plasmid is 2906

base pairs long and is maintained in *S. aureus* and *B. subtilis* at a copy number of 15 per cell. Deletion analysis (Alonso, 1987) has defined a 1.1-kilobase segment required for autonomous replication of the plasmid and a region adjacent to this that encodes a function necessary for stable inheritance in Rec⁺ cells. The basic replicon encodes positive, RepH, and negative, IncA, *trans*-acting products and their target sites. The *repH* gene product has a molecular mass of 34,100 and binds to the pC194 replication region. By *trans* complementation studies Alonso (1987) has shown that plasmid replication is controlled negatively at the level of RepH synthesis by the product of the *incA* gene that is located within the *repH* gene but is transcribed in the opposite orientation. Antisense RNA may regulate translation of *repH* by a mechanism involving RNA–RNA interaction.

4. PLASMID STABILITY

Plasmid instability has been a major problem associated with the successful cloning of genes in *B. subtilis*. Two types of instability have been described; *segregational instability* involving the loss of the entire plasmid and *structural instability* involving the loss, rearrangement, or acquisition of DNA by the plasmid.

4.1. Structural Instability

Structural instability may arise by deletion, insertion, or rearrangement of DNA. The best documented cause of structural instability is the deletion of plasmid DNA arising from recombination between short direct repeats. Some of the earliest reports of deletions were in bifunctional or shuttle plasmids which replicate in both *E. coli* and *B. subtilis* (for reviews, see Kreft and Hughes, 1982; Ehrlich *et al.*, 1982; Chapter 6, Section 3.8). Spontaneous deletions have now been detected in a wide range of plasmid, viral, and chromosomal DNAs. It has been postulated that *B. subtilis* has a very efficient recombination machinery and that plasmid deletants may in some instances rapidly predominate when competing with larger plasmids. Two basic types of rearrangement occur by either inter- or intramolecular recombination.

4.1.1. Intermolecular Recombination

Intermolecular rearrangements include *recE*-dependent integration of homologous DNA sequences into the *B. subtilis* chromosome (Aubert *et al.*, 1982). Homology between plasmid and chromosomal sequences results in intermolecular recombination and the integration of plasmid DNA into the chromosome and these events have been exploited to develop a series of integration vectors (see Chapters 4 and 6). Linearized plasmid DNA transformed into *B. subtilis* is not normally established and maintained. However, if the competent cells contain a homologous plasmid, then recombination between the homologous region can rescue sequences on the linear DNA (Gryczan *et al.*, 1980).

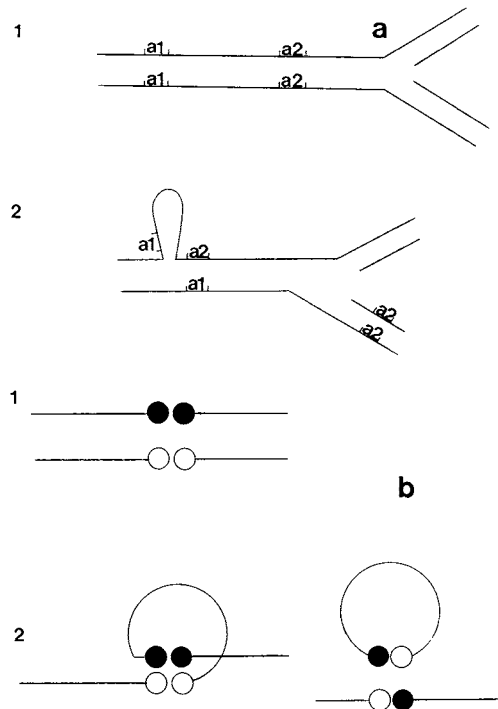
Intermolecular recombination is more frequent in plasmids introduced into cells by competent cell transformation than by protoplast transformation methods.

Michel and Ehrlich (1984) transformed *B. subtilis* and *E. coli* cells with plasmids unable to replicate in these bacteria but which carried sequences of varying lengths of homology with a resident plasmid. The recombination efficiency between resident and transforming plasmids varied with the square of the length of the homologous sequences carried by the two plasmids in three types of cells, competent *B. subtilis* cells, competent *E. coli* cells, and *B. subtilis* protoplasts. Recombination in competent *E. coli* cells was 100 times less efficient than in *B. subtilis* protoplasts and 10,000 times less efficient than in *B. subtilis* competent cells. These differences may reflect the different ways in which DNA is processed during transformation.

4.1.2. Intramolecular Recombination

Intramolecular rearrangements are often independent of the general recombination systems of the host strain. Short regions of homology in direct orientation can lead to recombination in *E. coli*. In *B. subtilis*, *recE*-independent events are not as well characterized but homologous DNA sequences have been implicated in such intramolecular events (Tanaka, 1979). Hahn and Dubnau (1985) ligated *S. aureus* plasmids pSA2100 (7.1 kilobase pairs) and pUB110 (4.5 kilobase pairs) at their unique *Xba*I sites and transformed into either Rec⁺ or *recE4* strains of *B. subtilis*. An intramolecular recombination event occurred in both backgrounds generating two plasmids, one of 2.6 kilobase pairs, the other of 9 kilobase pairs. This reciprocal *rec*-

Figure 3. (a) A model for deletion formation by slipped mispairing. During DNA replication slipped mispairing, which may be enhanced by inverted repeats between the direct repeats a1 and a2, may occur. Deletion formation results either from replication through the structure illustrated in 2 or from excision repair of the looped region. (b) A model for gyrase-mediated recombination. Circles represent the $\alpha\beta$ form of DNA gyrase composed of one gyraseA promoter and one gyraseB promoter. Two adjacent circles constitute the $\alpha_2\beta_2$ form, which is a putative gyrase structure. 1, DNA gyrase binds to DNA and transiently cleaves double strands resulting in an intermediate structure in which gyrase is covalently bound to each of the 5' termini of the DNA. Exchange may occur between $\alpha\beta$ subunits. 2, Intramolecular exchange of subunits may lead to deletion of DNA.



independent event occurred between sites containing a 34-base pair region of imperfect homology (34/36) on each plasmid. Two models have been proposed for such *recA*-independent deletion formation in *E. coli* and may be relevant here. Albertini *et al.* (1982) proposed that deletions may be generated by slipped mispairing of short homologies during replication (Fig. 3a) whereas Ikeda *et al.* (1981) proposed a DNA gyrase exchange mechanism. In the latter model DNA gyrase cleaves double-stranded DNA and becomes transiently bound to the 5' end. Two such DNA-gyrase complexes might reassemble with one another with an exchange of gyrase subunits and this may lead to deletion if the complexes form on a single DNA molecule (Fig. 3b). This model does not require DNA sequence homology for deletion to occur, only sequences which DNA gyrase recognizes and to which it binds.

4.1.3. Involvement of Replication in Recombination

It has been postulated that plasmid replication can stimulate recombination. The effect of replication on recombination between short sequences was investigated by inserting the thermosensitive plasmid pE194 into the *B. subtilis* chromosome (Ehrlich *et al.*, 1986). The frequency of recombination between a 4-kilobase pair long duplicated sequence flanking pE194 was close to 1 in 10^4 per cell generation when pE194 replication was inhibited by the restrictive temperature and greater than 1 in 10^2 per cell generation at the permissive temperature. The activity of the replication region stimulated recombination about 100-fold. The level of stimulation was dependent on the relative orientation of the replication region and the duplications. A 40-fold stimulation was observed for one orientation an 800-fold stimulation for the other. The putative effect of replication on recombination was therefore directional.

It has been proposed that single-stranded DNA can initiate recombination. If this initiation event is the rate-limiting step, it follows that the more single-stranded DNA present in a cell, the higher its recombination potential. Te Riele *et al.* (1986) observed that pC194 exists in both double- and single-stranded forms within *B. subtilis* and *S. aureus*. The single-stranded form was found as a circular molecule corresponding to only one of the two DNA strands. Single-stranded copies were observed for five other *S. aureus* plasmids, pC221, pC223, pE194, pT127, and pT181, and one *Bacillus* plasmid, pHV416. *Staphylococcus aureus* plasmid pUB110 and *B. cereus* plasmid pBC16 were, in contrast, mainly found as duplexes. From these results it is clear that a large amount of single-stranded DNA is generated during replication of a number of plasmids often used for cloning in *B. subtilis*. A model for the involvement of single-stranded DNA in recombination has been proposed by Ehrlich *et al.*, (1986), which is akin to the "slipped mispairing" model proposed by Albertini *et al.* (1982).

4.1.4. Instability Mediated by Insertion Sequence-like Elements

As well as homologous recombination between sites on a plasmid, structural instability may be mediated by insertion sequence (IS) elements or transposons resident in the host chromosome or on plasmids. These elements can cause insertion, adjacent deletion, or inversion mutations.

Alonso and Trautner (1985) described a derivative of pC194, pC194-1, in which a high frequency of deletions occurred in heterologous DNA ligated within the plasmid. The generation of these deletions was independent of *recE* and occurred at a 1000-fold higher frequency in *B. subtilis* than in *E. coli*. It was proposed that the base changes characterizing pC194-1 stabilized a potential stem loop structure within the plasmid that may be involved in deletion generation. Deletion formation in the pC194-1/pBR322 hybrid pHV15-1 was more pronounced at lower temperatures. This is reminiscent of the induction of deletions by IS1 in *E. coli* (Reis and Saedler, 1975). From a study of deletion end points within the pBR322-derived region of pHV15-1, it was proposed that homology with the sequence of the pC194-1 stem structure was involved in deletion formation.

4.2. Segregational Instability

The loss of a entire plasmid from its host is termed segregational instability. It has been proposed that naturally occurring, low-copy-number plasmids are stably maintained by partitioning (*par*) functions, which ensure that they are accurately segregated at cell division. *Par* functions have been characterized for the gram-ve plasmids pSC101 and R1 (Meacock and Cohen, 1980; Nordstrom *et al.*, 1980) and for the *B. amyloliquefaciens* vector pLS11 (Chang *et al.*, 1987). High-copy-number plasmids such as pBR322 do not normally possess *par* function and are segregated randomly at cell division. Under these conditions the probability $p(0)$ of either daughter cell failing to inherit a plasmid at cell division is given by the binomial distribution:

$$p(0) = 2(0.5)^c$$

where c is the number of plasmid molecules per cell. For a plasmid with 20 copies per cell, the probability of obtaining plasmid-free cells is 1.75×10^{-6} . For pBR322 the probability of plasmid-free cells arising is very low. However, under certain conditions, (e.g., nutrient limitation or during rapid cell growth) plasmid-free cells may arise (Jones *et al.*, 1980).

Plasmid instability may also arise as a result of multimeric forms of a plasmid. The mechanism which controls the copy number of a plasmid ensures a fixed number of plasmid origins per bacterium. Cells containing multimeric plasmids have the same number of plasmid origins but fewer plasmid molecules, which leads to greater instability if these plasmids lack a partitioning function. These multimeric forms are not seen with the naturally occurring ColE1, which has a mechanism of resolving multimers to monomers. The resolution site of ColE1 has been cloned into pBR322-type plasmids and has eliminated problems of segregational instability due to multimerization (Summers and Sherratt, 1984).

4.2.1. Effect of Size on Plasmid Stability

The effect of plasmid size on instability was investigated by inserting homologous *B. subtilis* or heterologous *E. coli* DNA into pUB110-derived vectors (Bron and Luxen, 1985). DNA was inserted into both the pUB110-derived plasmid pLB2 and the bifunctional replicon pLB5, which is able to replicate in *B. subtilis* and *E. coli*.

Irrespective of the source of the cloned DNA, the segregative instability of the recombinant plasmids in *B. subtilis* was severely affected by the size of the DNA inserts. No instability was observed in *E. coli*. In *B. subtilis* a steep inverse relationship existed between the size of the inserts and the level of stability. In exponentially growing cultures it was found that size markedly affected copy number in *B. subtilis*. In *E. coli* all recombinant pLB plasmids had similar copy numbers. If pUB110 does not have a partitioning mechanism or if it has been impaired, then a decrease in copy number may lead to plasmid instability. The reduced copy number of pLB derivatives may be due to impairment of the copy number control mechanism. The pUB110 segment present in the pLB plasmids lacks the membrane-binding regions BA3 and BA4 (see Section 3.1). Bron *et al.* (1987) reported that the deletion of the BA3 region from pUB110 leads to an increased amount of single-stranded form plasmid DNA, which they suggest may affect segregational as well as structural stability.

4.2.2. Partitioning Functions of *Bacillus* Plasmids

Problems of segregational instability among *Bacillus* plasmids have been largely overcome by using plasmids which contain a partition locus. The partitioning functions of two *Bacillus* plasmids, pLS11 and pTA1060, have been characterized.

4.2.2a. pLS11 and pSYC5191. pLS11 was isolated as an 8.4-kilobase pair cryptic plasmid from a strain of *B. subtilis* that has been subsequently reclassified as *B. amyloliquefaciens* (Tanaka *et al.*, 1977). The *ermC* gene from pE194 was cloned into pLS11 to generate pSYC5191 (Chang *et al.*, 1987). A 1.2-kilobase pair region from pLS11 was shown to stabilize the unstable pUB110 derivative pOG2326 when cloned *in cis* but not *in trans*. This suggests that *par* does not specify a diffusible product and in this respect is analogous to the *par* regions of pSC101 and R1. The *par* region of pLS11 has been localized to a 167-base pair segment, sequencing of which reveals a single *Bst*EII site at position 147. Insertion into or deletion of sequences either side of this site inactivates the *par* locus. It is proposed that *par* is a site of interaction with other cellular components. This interaction is most likely linked to plasmid replication since it functions in only one of the two possible orientations relative to the replication origin (Chang *et al.*, 1987). The 147-base pair *Hae*III to *Bst*EII fragment of pLS11 *par* has been used as a probe in southern analyses for other *par* sequences. However, no homology was found with chromosomal DNA from *B. subtilis*, *B. amyloliquefaciens*, and *E. coli* (Chang *et al.*, 1987). It is unlikely that *par* functions through recombination with the chromosome.

4.2.2b. pTA1060. pTA1060 was isolated from a strain of *B. subtilis* (Bron *et al.*, 1987). pBB2, a chloramphenicol and kanamycin-resistant derivative of pTA1060, was almost completely stable during 100 generations of cell growth under nonselective conditions. A 1.45-kilobase pair *Cla*I fragment of pTA1060 was found to be important for the high level of stability. The insertion of a 4.2-kilobase pair fragment of DNA into pBB2 reduces stability only slightly. The same DNA inserted into the pUB110-derived pLB plasmids dramatically reduced stability (Bron and Luxen, 1985). pTA1060 may therefore provide the basis of a series of vectors for the cloning of relatively large inserts in *B. subtilis*.

5. METHODS FOR THE DETERMINATION OF PLASMID COPY NUMBER

Three methods of copy number determination have been developed based on either the physical separation of plasmid from chromosomal DNA, hybridization techniques, or the measurement of gene dosage.

5.1. Physical Separation Techniques

For this type of technique plasmid DNA is separated from chromosomal DNA either by density gradient centrifugation or by agarose gel electrophoresis.

5.1.1. Density Gradient Centrifugation

Total cell DNA is radiolabeled and plasmid DNA present in its covalently closed circular (CCC) form is separated from chromosomal DNA by cesium chloride–ethidium bromide gradient centrifugation (Bazaral and Helinski, 1968). Using this technique the plasmid copy number is determined by calculating the ratio of supercoiled DNA to chromosomal DNA. If the chromosomal size is known, then copy number is expressed as copies per chromosome equivalent. This gives only a minimum estimate of copy number since it does not allow for loss of DNA during extraction or for non-CCC forms of the plasmid.

5.1.2. Agarose Gel Electrophoresis

Weisblum *et al.* (1979) developed a method in which radiolabeled total cell DNA is separated using agarose gel electrophoresis. Gel bands corresponding to chromosomal and plasmid DNA are excised from the gel and their radioactivity measured. From the ratio of plasmid to chromosomal DNA and a knowledge of the size of the bacterial chromosome a copy number estimate is obtained.

As a modification of this technique, densitometer scanning of photographic negatives of gels stained with ethidium bromide has also been used (Primrose *et al.*, 1983). More recently, Projan *et al.* (1983) refined the technique further by using direct fluorescence densitometry of stained gels. The direct scanning of gels has an advantage over densitometry of photographic negatives in that the latter depends on linearity of both fluorescent signal and film response.

5.2. Hybridization Techniques

Hybridization methods are more precise but are difficult to perform. These methods require total cell DNA to be fixed to nitrocellulose filters and the subsequent annealing of radioactive plasmid DNA (Shepard *et al.*, 1979). Copy numbers calculated from hybridization data were found to be significantly higher than those from dye-buoyant density profiles (Mayer *et al.*, 1977).

Recently, Nyberg *et al.* (1985) described a method based on a sandwich hybridization technique. The DNA fragment to be measured is trapped between two nonoverlapping fragments each of which have homology with different regions

of the test fragment. The fragments are cloned into different, nonhomologous vectors. One fragment is attached to a nitrocellulose filter and is hybridized with the test DNA fragment. The other fragment is radiolabeled and is used as a probe which can hybridize with the test fragment but not with the fragment fixed to the filter. The amount of radioactivity bound to the filter gives a measure of the copy number of the test fragment. Using this method there is no need for labeling of the test fragment. The copy number of plasmids grown in any media can therefore be obtained. Nyberg *et al.* (1985) used this technique to determine the copy number of the pUB110 derivative pKTH10, which contains the α -amylase gene from *B. amyloliquefaciens*. The copy number of pKTH10 was of a different magnitude from previous estimates and ranged from 2500 copies per cell in early logarithmic growth to 500 in late stationary phase.

5.3. Gene Dosage Methods

These methods require that the phenotype being measured is linearly in proportion to gene dosage (Uhlen and Nordstrom, 1977). This method is therefore limited to plasmids that contain one of the few genes whose products show the linear proportionality.

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Sporulation and Germination

8

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1. INTRODUCTION

Since the 1950s bacterial sporulation in *Bacillus* has been recognized as a model system for analyzing differentiation in a prokaryotic organism. Earlier studies focused on the physiological and morphological changes which occurred during conversion of the vegetative cell into a heat-resistant, dormant spore. The physiological and morphological studies were complemented by the isolation of mutants which developed up to specific recognizable stages and were blocked in further development. However, the nature of spore-forming bacteria and the absence of suitable genetic and technical approaches precluded the precise analysis of the regulatory mechanisms which controlled sporulation. It was not possible to determine (1) which events regulated the initiation of sporulation, (2) what products were encoded by the sporulation and germination genes, (3) how genes were expressed in a temporally regulated fashion, (4) what the relationship was between catabolite repression and the initiation of sporulation, (5) what temporally regulated genes were essential for sporulation, (6) what transcriptional mechanism was required for the expression of sporulation genes, and (7) how the morphologically complex spore structure was assembled.

With the recent advent of recombinant DNA technology, the investigation of the regulatory events and the mechanisms of sporulation and germination in *Bacillus subtilis* has taken a dramatic upswing. Although the questions posed above have not been answered to date, the rapid progress being made currently will surely provide numerous answers and fulfill the expectations of those investigators who initiated the earlier studies on sporulation.

The earlier basic information concerning sporulation and germination is being complemented by information obtained by the new technology to provide a better understanding of the regulation of gene expression during growth and development.

Rapid progress has been made in the cloning and characterization of sporulation genes (*spo*), the isolation and characterization of forespores, the determination of the properties and specificities of RNA polymerase holoenzymes, the under-

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standing of the factors which affect the initiation of sporulation, and the elucidation of the relationship between *spo0* genes, RNA polymerase, and catabolite repression. This chapter reviews and discusses some of these recent advances along with related information that can be utilized for further basic and applied research. A particular emphasis will be placed on evaluating our current understanding of the regulatory mechanisms which control the initiation of sporulation.

Several reviews which are pertinent to this discussion include the compilation of the latest genetic linkage map of *B. subtilis* (Piggot and Hoch, 1985; Zeigler and Dean, 1985); a summary of sporulation and germination genes (Piggot and Hoch, 1985); a review of the genetics of germination (Moir *et al.*, 1985); a description of several new methods to study developmental genes (Youngman *et al.*, 1985); a discussion of temporal gene expression in *B. subtilis* (Doi, 1984); and analysis of the initial events of bacterial sporulation (Freese, 1981); a description of the biochemistry of sporulation and germination (Setlow, 1981); and a discussion of the genetics of sporulation and germination (Piggot and Coote, 1976; Losick *et al.*, 1986).

2. PHYSIOLOGICAL AND MORPHOLOGICAL CHANGES ACCOMPANYING SPORULATION AND GERMINATION

A brief description of the physiological and cytological changes which occur during sporulation is presented as a background to the discussion of the various regulatory mechanisms currently being analyzed.

2.1. Response to Nutrient Deprivation

Nutrient deprivation of *Bacillus* triggers several cellular responses that attempt either to prevent starvation or to prepare the cell for a long period when nutrients are not available. The lack of readily metabolizable carbon, nitrogen, and phosphorus compounds are known to induce these events (Schaeffer *et al.*, 1965b). One early response of the cell to lowered concentrations of nutrients is to synthesize flagella which convert essentially nonmotile, linked, logarithmic phase cells into highly motile, individual, early stationary phase cells. This increased motility allows the cell to search for nutrients and respond chemotactically to a source of nutrients (Ordal *et al.*, 1985).

In addition to increased motility, the absence of nutrients derepresses a number of genes which code for intracellular and extracellular enzymes. The intracellular enzymes include enzymes of the tricarboxylic acid (TCA) cycle (Hanson *et al.*, 1963) and other carbon utilization enzymes (Nihashi and Fujita, 1984). When *Bacillus* is grown on glucose, organic acids such as pyruvate and acetate accumulate in the medium and reduce the pH of the medium to about 5–6 (Nakata and Halvorson, 1960). After glucose is depleted and the TCA enzymes are derepressed, the organic acids are utilized as a source of carbon and the pH of the medium rises to about 7. Thus a readjustment of the intracellular metabolic enzymes allows the cell to utilize other sources of carbon.

Besides synthesizing a new array of intracellular enzymes, the genes for extracellular enzymes are derepressed during nutrient starvation. These secreted en-

zymes include proteases, nucleases, amylases, phosphatases, and other hydrolytic enzymes (Schaeffer, 1969; see Chapter 11). These extracellular enzymes search out and attack suitable biopolymers and substrates in the microenvironment and furnish the cell with suitable monomeric nutrients.

If the cell is successful in swimming to another source of nutrients or its extracellular enzymes are able to hydrolyze sufficient amounts of nutritious biopolymers in its microenvironment, then the cell continues to grow. However, if these initial responses fail to provide the cell with required nutrients, the cell then initiates another sequence of events which results in the formation of a dormant spore. Spores are capable of remaining dormant for many years under the appropriate conditions; however, these same spores can germinate within minutes when suspended in a suitable nutrient-rich environment.

Although sporulation is a dramatic and morphologically identifiable stage in the life cycle of *Bacillus*, it could be considered as a "last resort" response of the cell to the very stressful situation of nutritional deprivation. Many bacteria also respond to starvation by growing flagella and derepressing genes for catabolic functions, but do not sporulate. Many of these bacteria live in a less hostile environment than species of *Bacillus* which tend to live in nutritionally poor environments such as soil and dry grass. The special ability of *Bacillus* species to sporulate allows these microorganisms to tolerate exposure to poor nutritional conditions for prolonged periods of time.

2.2. Sporulation Stages

Sporulation has been divided into seven stages based on the cytological changes which occur during spore formation (Schaeffer *et al.*, 1965a; Ryter *et al.*, 1966) (Fig. 1). Stage 0, which is a preparatory stage for sporulation, occurs at the end of the logarithmic phase of growth or at T_0 (T_1 , T_1 , T_2 , T_3 , etc., represent the time in hours after the end of the log phase). The sporulation process usually takes about 6–8 hr under laboratory conditions of growth and thus the stage of development and the time in hours is rather similar, e.g., stage IV occurs at about T_4 .

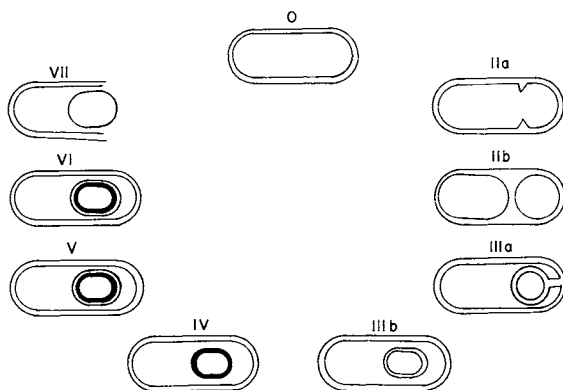


Figure 1. Schematic illustration of sporulation stages in *B. subtilis*. Proceed clockwise from stage 0, which occurs at the end of the logarithmic phase of growth. See text for description of stages.

At stage 0 no morphologically discrete sporulation structure is present and the cell has an appearance similar to that of a vegetative cell. Currently, stage I is no longer used to define a morphological state during sporulation; thus the developing cells go from stage 0 to stage II.

Stage II is the first sporulation stage that is morphologically identifiable and is characterized by the appearance of a forespore septum which divides the cell asymetrically into a small forespore and a larger mother cell. This is a critical step, since asymmetrical membrane formation appears to be a key step in the development of sporulation.

During stage III the mother cell engulfs the forespore. The forespore, which now resides in the cytoplasm of the mother cell, has a double-membrane structure in which molecules traveling from the cytoplasm of the mother cell to the cytoplasm of the forespore would pass through a double membrane via the following path:

Mother cell	outer forespore		inner forespore	forespore
cytoplasm	→ membrane	→ space	membrane	→ cytoplasm
	(inside-to-outside)		(outside-to-inside)	

The outer forespore membrane has an opposite polarity to the inner forespore membrane. The "inside out" nature of the outer forespore membrane has been demonstrated with isolated forespores which can readily oxidize NADH (Andreoli *et al.*, 1975) and hydrolyze ATP (Ellar *et al.*, 1975; Wilkinson *et al.*, 1975), when these substrates are provided in the medium.

After engulfment the cell is committed to sporulation and enrichment of the medium at this point will not reverse the sporulation process (Freese *et al.*, 1970).

Cortex formation is initiated between the inner and outer forespore membranes during stage IV at which time the developing spore becomes refractile. Cortical development probably continues into the next stage.

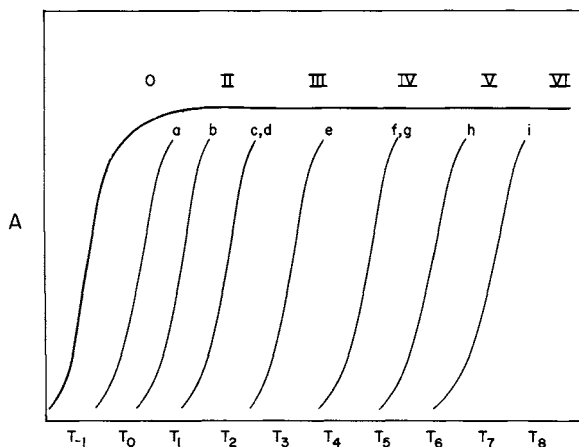
Stage IV is followed by inner spore coat protein deposition on the surface of the outer forespore membrane during stage V. The inner spore coat is complex and consists of a multilayered laminar structure.

The outer spore coat is deposited on the surface of the inner spore coat during stage VI. The spore coat is very complex and consists of a proteinaceous outer coat layer and a multilayered laminated inner spore coat. As the spore coats develop the spore becomes increasingly resistant to chemicals and to heat. Spore maturation occurs during stage VI and is followed finally by release of the mature spore from the mother cell at stage VII.

Several developmental mutants have been isolated which are blocked at these various morphological states and the mutations have been designated as *spo0*, *spoII*, *spoIII*, *spoIV*, *spoV*, and *spoVI* depending on the last morphological stage of development which can be observed in these mutants.

The exact timing of sporulation under laboratory conditions depends on the strain of *Bacillus* and the medium used. With the *B. subtilis* Marburg strain and a modified Schaeffer medium (Leighton and Doi, 1971) sporulation is initiated at T_0 and by T_5 a refractile spore can be observed in the mother cell. A similar pattern of sporulation is observed when cells are shifted from a rich growth medium to a more defined minimal resuspension medium (Sterlini and Mandelstam, 1969). Sporulation occurs most synchronously and efficiently when cells have grown in a rich

Figure 2. Schematic illustration of marker events during sporulation of *B. subtilis*. A, the absorbance of the culture. T, time in hours starting with T₀ at the end of the exponential phase of growth. (a) Motility. (b) Competence. (c) Serine protease. (d) Spore coat precursor proteins. (e) Alkaline phosphatase. (f) Glucose dehydrogenase. (g) Dipicolinic acid (DPA) synthesis. (h) Small acid-soluble proteins (SASPs). (i) Spore coat proteins. The Roman numerals indicate the stages of sporulation. See text for a discussion of the occurrence of these events.



medium for a minimum of six to seven generations prior to the onset of sporulation.

The appearance of particular enzymes and functions has been associated with specific stages of sporulation (Warren, 1968; Kay and Warren, 1968; Waites *et al.*, 1970); it is now clear that some of these enzymes are not directly involved in the sporulation process. This has been demonstrated by making deletion mutations in the genes of these enzymes and testing for sporulation. In the case of subtilisin and neutral protease, sporulation continued normally in the complete absence of these enzymes (Kawamura and Doi, 1984; Yang *et al.*, 1984). It is possible that not all the extracellular enzymes which are synthesized during sporulation are required for the sporulation process, although their synthesis may be in part under similar control as that of genuine sporulation products. Still, as readily identifiable landmarks of the sporulation process, it is useful to keep these enzymes and their functions in mind. The occurrence of these activities in relationship to the growth phase is illustrated in Fig. 2.

2.3. Pattern of Protein Synthesis

The sporulation process is accompanied by dramatic morphological as well as functional changes. The overall pattern of protein synthesis during sporulation has been investigated by comparing the electrophoretic patterns of proteins synthesized during vegetative growth and sporulation (Linn and Losick, 1976; Andreoli *et al.*, 1978; Yudkin *et al.*, 1982a,b; Boschwitz and Yudkin, 1983). Several patterns have been noted including the decline in some vegetative proteins during sporulation, the continued presence of some vegetative proteins at the same level, and the appearance of new proteins during the early and later stages of sporulation.

The proteins which appear specifically during the early part of the stationary

phase are of particular interest, since their appearance indicates that either catabolite-repressed or specific developmental genes have been expressed. The expression of catabolite-repressed genes allows the cell to reorient its metabolism, e.g., by activating the tricarboxylic acid cycle (Hanson *et al.*, 1964) or by secretion of extracellular enzymes (Schaeffer, 1969). Release from catabolite repression is therefore one of the earliest responses to nutrient deprivation. One of the shortcomings of looking at overall protein patterns is that they can demonstrate only the relative extent of changes which are occurring. Specific protein changes cannot be identified unless the protein spots have been identified previously, as is the case in *E. coli* (Neidhart *et al.*, 1983). Unfortunately, this is still not the case for *B. subtilis* proteins.

2.4. Separation of Forespore and Mother Cell

A more specific way to study the protein patterns of sporulating cells is to examine the proteins present in the mother cell and in the forespore. This should provide information on the location of particular proteins during the differentiation process. For this purpose a method is required for isolating intact forespores from the mother cell after the engulfment stage so that comparisons may be made between their protein patterns. However, one of the difficulties with analyzing the proteins of isolated forespores is that it cannot be said for certain whether the genes for these proteins were expressed in the forespore or in the mother cell prior to forespore formation (stage 0). In the latter case the proteins would be entrapped in the forespore during foreseptum formation and subsequent engulfment. Also, it is possible that a protein synthesized in the mother cell could be transported across the double membrane into the forespore. Although these limitations exist, several investigators have obtained data which suggest that both the mother cell and the forespore chromosomes are actively and differentially expressed during sporulation.

3. SPORULATION-RELATED PROTEINS OF THE FORESPORE AND MOTHER CELL

The specific types of proteins which have been investigated as they relate to the sporulation process include various intracellular enzymes, membrane proteins, spore coat proteins, small acid-soluble spore proteins, and extracellular enzymes.

3.1. Forespore and Mother Cell Proteins

The initial morphological evidence for the onset of sporulation is associated with the asymmetrical synthesis of a forespore membrane during stage II. This process leads ultimately to the compartmentalization of the cell into a small forespore nestled within the mother cell (stage III or engulfment). Cellular events prior to stage II control this important step, since *spo0* mutants (blocked at stage 0) do not form a forespore membrane (Ryter *et al.*, 1966).

There has been a question for some time as to whether the forespore is differentially expressing genes for specific sporulation functions during its maturation

process (Warren, 1968; DeLencastre and Piggot, 1979). This question has been partially answered by the development of suitable methods for the isolation of intact forespores at various stages of sporulation (Andreoli *et al.*, 1973; Ellar and Postgate, 1974). The availability of intact forespores has allowed a comparison of the enzymes and proteins synthesized within the mother cell and forespore cytoplasm.

The isolated forespore is able to synthesize proteins *de novo* as demonstrated with forespore extracts and intact forespores (Andreoli *et al.*, 1975; Watabe *et al.*, 1981); moreover, more RNA synthesis occurs per unit of cell weight in the forespore than in the mother cell (Nakayama *et al.*, 1980). Although there is a high rate of protein turnover in the mother cell (Kornberg *et al.*, 1968), no protein turnover was detected in the forespore (Eaton and Ellar, 1974).

The data obtained by two-dimensional isofocusing-sodium dodecyl sulfate polyacrylamide gel electrophoresis (O'Farrell, 1975) indicate that the pattern of proteins in the early forespores resembles that found in the mother cell, but as the forespore matures, the patterns found in the two cytoplasmic compartments diverge. Some genes are expressed in both compartments, while others are expressed either in the mother cell or in the forespore. These results are summarized in Table I.

Some spore components such as alanine dehydrogenase are synthesized in the mother cell compartment and then are transported to the developing spore—perhaps to its outer surface layers (Andreoli *et al.*, 1973). The enzymes which synthesize dipicolinic acid are found only in the mother cell, but dipicolinic acid is

Table I. Site of Protein Synthesis in the Mother Cell and Forespores^a

Protein	Mother cell	Forespore	References
Alanine dehydrogenase	+	—	Andreoli <i>et al.</i> , 1973
Dipicolinic acid synthetase	+	—	Andreoli <i>et al.</i> , 1975
Dihydrodipicolinic acid synthase	+	—	Andreoli <i>et al.</i> , 1975
Quinolinate phosphoribosyl-transferase	+	—	Andreoli <i>et al.</i> , 1975
Spore coat precursor	+	—	Munoz <i>et al.</i> , 1978; Aronson and Pandey, 1978; Jenkinson <i>et al.</i> , 1981
Alanine aminotransferase	+	+	Andreoli <i>et al.</i> , 1973
Aspartate aminotransferase	+	+	Andreoli <i>et al.</i> , 1973
NADH oxidase	+	+	Andreoli <i>et al.</i> , 1973
NADH-DCIP ^b oxidoreductase	+	+	Andreoli <i>et al.</i> , 1973
Small acid-soluble spore proteins (SASPs)	—	+	Singh <i>et al.</i> , 1977; Tipper <i>et al.</i> , 1981
SASP protease	—	+	Singh <i>et al.</i> , 1977
Glucose dehydrogenase	—	+	Fujita <i>et al.</i> , 1977; Nakayama <i>et al.</i> , 1980; Chaudry <i>et al.</i> , 1984

^a + means that synthesis occurs and — means synthesis does not occur in the cell compartment. Many, but not all, of these data are based on the analysis of isolated forespores.

^bDCIP = 2,6 dichlorophenol indophenol.

found only in the dormant spore (Andreoli *et al.*, 1975). Thus small metabolites appear to be able to cross the double membrane of the developing forespore.

Since spore coat precursor proteins are made prior to engulfment (stage III), it is likely that spore coat proteins are synthesized only in the mother cell compartment and processed either during or after they have been transported to the outer surface of the maturing spore (Munoz *et al.*, 1978; Aronson and Pandey, 1978; Pandey and Aronson, 1979). Other proteins, such as glucose dehydrogenase (Fujita *et al.*, 1977; Nakayama *et al.*, 1980) and small acid-soluble spore proteins (SASPs) (Singh *et al.*, 1977; Tipper *et al.*, 1981), are synthesized only within the forespore. The RNA polymerase σ factor (Haldenwang *et al.*, 1981; see Section 6.1) is synthesized as early as T₂ in the mother cell (Fukuda and Doi, 1977) but is found primarily within the forespore cytoplasm (Nakayama *et al.*, 1980). Since this protein is synthesized in a precursor form (Trempy *et al.*, 1985a; LaBell *et al.*, 1987), it is possible that the precursor form facilitates its transport into the forespore from the mother cell cytoplasm. Thus these examples indicate strongly that differential gene expression occurs within both the mother cell and forespore compartments during sporulation and that some type of developmental mechanism regulates the final physical disposition of the protein products. The elucidation of this latter mechanism will test the imagination and skill of future experimenters.

Regulation of Activity of Forespore Enzymes

Mechanisms exist in the forespore which regulate the activity of its enzymes. One example which has been noted is the synthesis and accumulation of SASPs, which are synthesized during stages IV–V at the same time as the specific SASP protease (Singh *et al.*, 1977). The SASP protease efficiently degrades SASPs during germination (Setlow, 1975a), but is obviously not active during sporulation when large amounts of SASPs are synthesized and stored in the developing spore.

Another case involves the synthesis and storage of high levels (up to 5% of spore dry weight) of 3-phosphoglyceric acid (3-PGA) in the developing spore. In some *Bacillus* species 3-PGA is one of the main energy reserves of the dormant spore (Setlow, 1981). During germination 3-PGA is utilized in the first 10–15 min to yield energy and acetate for the germinating cell. This compound is accumulated during sporulation in the presence of significant levels of the enzymes which can catabolize it. Thus, as with the SASPs, 3-PGA must be inaccessible to phosphoglycerate mutase and the other degradative enzymes (Singh and Setlow, 1979a,b). Therefore mechanisms exist in the developing forespore which either inhibit the activity of the various catabolic enzymes or separate the various enzymes from their substrates by some type of compartmentalization until the germination mechanism is activated.

3.2. Forespore Septum Proteins

Since the synthesis of the forespore septum plays a critical role in the initial stages of sporulation, it is highly likely that it has some unique features which distinguish it from the mother cell cytoplasmic membrane.

By use of two-dimensional electrophoresis (O'Farrell, 1975), Andreoli *et al.*

(1981) demonstrated the presence of at least 260 membrane proteins in late exponential and stage III (engulfment) cells. Approximately 50 proteins present in stage III cell membranes were absent from the membranes of late exponential phase cells. In comparing specific membrane proteins they observed a series of protein increases, decreases, deletions, and the appearance of totally new proteins. Many of the new proteins were already present in the forespore septum (stage II) and very few new proteins were added to the membrane during the engulfment process (stage III).

Subsequently, Chui *et al.* (1984) and Andreoli (personal communication) found six new major membrane proteins that appeared during asymmetrical septum assembly and two new major proteins were observed only during engulfment membrane formation. The membrane of a *spoIIA* mutant, which forms an asymmetrical septum but fails to assemble engulfment membranes, contained only the six membrane proteins found in the asymmetrical septum and lacked the two engulfment membrane proteins. The occurrence of the eight new major membrane proteins was independent of the conditions used for obtaining sporulation. Thus these observations indicate that specific proteins may be required for the formation of the asymmetrical septum and for engulfment of the forespore.

In this regard, studies on intergenic suppression of *spo0* mutants (Sharrock *et al.*, 1984; Leung *et al.*, 1985) and the partial homology of the *spo0A* protein product to the *ompR* gene product (Ferrari *et al.*, 1985b) indicate that membrane functions may play key roles during forespore septum formation and the initiation of sporulation. Wayne *et al.* (1981) and Wayne and Leighton (1981) showed that mutationally induced *Spo^{ts}* phenotypes and the cerulinin-induced *Spo^{ts}* phenocopy (cerulinin is an inhibitor of fatty acid synthesis) can be relieved by the presence of precursors of membrane biosynthesis. Also, a suppressor mutation *rev4* can restore sporulation in *Spo^{ts}* mutants, in *Spo^{ts}* phenocopies induced in wild-type *B. subtilis* by ethanol and phenethyl alcohol, and in cerulinin-treated wild-type cells (Sharrock and Leighton, 1982). Therefore the *rev4* suppressible sporulation phenotypes appear to be associated with defective membrane structure or function.

Another intergenic suppressor of *spo0* phenotypes called *rvtA* (Sharrock *et al.*, 1984) can prevent the disruption of sporulation by aliphatic alcohols in a manner similar to that of *rev4*. Since *rvtA* is now considered to be a mutation in the *spo0A* gene (Ferrari *et al.*, 1985b), it appears that *spo0* genes are involved in controlling membrane functions.

The further characterization of the sporulation-related membrane proteins and their genes should shed light on their role in the assembly and functions of forespore septum and engulfment membrane. Furthermore these studies should clarify the role, if any, of *spo0A* on the regulation of membrane protein genes.

3.3. Spore Coat Proteins

One of the ironies of studying sporulating cells has been the difficulty in identifying sporulation-specific proteins. Many activities associated with sporulation may turn out to play no direct role in sporulation itself. However, the spore coat proteins are obviously good candidates for study as sporulation-specific gene products, since their genes are expressed only during sporulation and their products are

clearly spore components. The coat proteins may comprise from 40 to 80% of the total spore protein (Spudich and Kornberg, 1968; Munoz *et al.*, 1978; Goldman and Tipper, 1978; Aronson, 1981) and therefore are major components of the spore and major products of the protein-synthesizing machinery of the sporulating cell.

There has been, however, some difficulty in isolating and characterizing spore coat proteins since they tend to be insoluble and require rather harsh extraction treatments (Pandey, 1980). The total extraction of spore coat proteins requires a combination of detergents, protein-denaturing agents, high pH conditions, and agents which disrupt disulfide bonds. Since these proteins are nonenzymatic, they are usually assayed immunologically, which may or may not be specific depending on the antigen preparation used for antibody production. In spite of these inherent difficulties, the spore coat proteins have been well characterized in recent years.

The spore coat is a complex structure consisting of at least 12 different polypeptides (Goldman and Tipper, 1978; Pandey and Aronson, 1979; Pandey, 1980; Jenkinson *et al.*, 1981) and is composed of several layers of proteins which are laid down successively during spore maturation (Jenkinson *et al.*, 1981; Jenkinson, 1981; Jenkinson and Sawyer, 1981).

At least some of the spore coat proteins are made as early as stage II, i.e., before forespore engulfment (Wood, 1972; Munoz *et al.*, 1978; Pandey and Aronson, 1979). They are made as precursor coat proteins (Munoz *et al.*, 1978; Pandey and Aronson, 1979), which are processed to the mature form presumably by specific intracellular proteases (Kerjan *et al.*, 1979; Srivastava and Aronson, 1981; Jenkinson and Lord, 1983) either before or after they are assembled on the surface of the developing spore. This makes the analysis of late sporulation processes somewhat more difficult, since some of the events occurring after stage IV require the processing and assembly of proteins formed earlier in stages II and III (Dion and Mandelstam, 1980; Jenkinson *et al.*, 1980). Also the later stage IV–VI phenotypes may be the result of a mutation which affects the synthesis of a protein as early as in stage II. Thus some caution must be taken in interpreting the phenotypes of so-called late stage mutants.

Several spore coat mutants have been found and designated as *gerE* (Moir *et al.*, 1979), *spoVIA* (Jenkinson, 1981), and *spoVIB* (Jenkinson, 1983). These mutants have abnormal spore coats, and may have altered heat resistance and germination properties. The *spoVIA* mutant spore lacks a 36,000-Da polypeptide normally found in the outermost layer of the mature spore, has a delayed response to germinants, and is sensitive to lysozyme. It is, however, fully heat-resistant (Jenkinson, 1981). The *spoVIB* mutant has a 12,000-Da protein which is assembled abnormally into the spore coat; this mutant sporulates and germinates more slowly than normal cells and is therefore a spore coat *assembly* mutant (Jenkinson, 1983). The *gerE* mutant produces spores that are resistant to heat and organic solvents, are lysozyme-sensitive, and have an incomplete coat structure (Moir, 1981). The coat lacks a number of polypeptides but has four polypeptides not present in wild-type spore coats (Jenkinson and Lord, 1983). The *gerE* mutant also fails to synthesize an intracellular protease B at stage V which is normally incorporated into spore outer layers. The highly pleiotropic nature of this mutation suggests that the *gerE* locus codes for a regulatory product required for expression of late sporulation genes (Moir, 1981).

The analysis of spore coat proteins presents several intriguing questions:

(1) How do the spore coat proteins made early in sporulation survive the rigorous turnover of proteins which occurs in the mother cell during sporulation? Are the precursor forms resistant to intracellular proteases? Do the precursors become susceptible to protease cleavage only after assembly on the surface of the maturing spore? Is there one or several proteases that can process the precursors?

(2) How are the spore coat proteins transported to the developing spore surface and when are the precursor coat proteins processed to the mature form? Since the mature forms are rather insoluble at neutral pH and in aqueous solutions, it appears likely that soluble precursors are assembled on the developing spore surface prior to final processing.

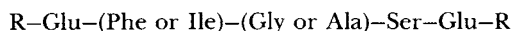
(3) What factors regulate the expression of the spore coat protein genes? Since some of these proteins are made very early in sporulation (stage II) and some are made later (stage V–VI) (Jenkinson and Sawyer, 1981), it is possible that different forms of the transcriptional machinery may transcribe these coat protein genes.

(4) What factors determine the order of assembly of the many layered spore coat complex? Is the time of synthesis of each of the spore coat proteins sufficient to determine the sequential layering of the spore coat proteins? Are there assembly proteins which facilitate the proper layering process? Is there sufficient information in the coat protein molecule itself to control the assembly process, i.e., does self-assembly occur?

Some of these questions will be answered soon, since spore coat protein genes have been cloned (Donovan *et al.*, 1987) and can be used as probes for measuring spore coat gene expression. The elucidation of the mechanisms for processing and the sequential assembly of the spore coat proteins on the surface of developing spores will require a substantial amount of effort. It would be quite a feat if an *in vitro* system could be developed to provide all the coat protein precursors, processing enzymes, and a forespore for the assembly of the spore coat complex perhaps in a manner analogous to that of the less complex phage particle.

3.4. Small Acid-Soluble Spore Proteins

The small acid-soluble spore proteins (SASPs), whose molecular masses range from 12,000 to 15,000, have been studied extensively by Setlow and his colleagues (Fliss *et al.*, 1985). These proteins, which comprise 10–20% of the dormant spore protein, are degraded during germination and provide amino acids for the germinating spore (Setlow *et al.*, 1980; Setlow, 1981; Fliss *et al.*, 1985). The spore protease which initially cleaves these proteins during germination has a very high sequence specificity and attacks a target site with the following amino acid sequence (Dignam and Setlow, 1980; Setlow *et al.*, 1980; Yuan *et al.*, 1981; Fliss and Setlow, 1984b):



The cleavage occurs between the Glu and (Phe or Ile) residues.

The SASPs are synthesized in the forespore throughout the sporulation period (Dignam and Setlow, 1980) and their expression is under transcriptional control (Setlow, 1981). Several SASPs have been completely sequenced (Setlow and Ozols, 1979, 1980a,b; Fliss and Setlow, 1985; Fliss *et al.*, 1986; Connors *et al.*, 1986). Seven

SASP genes have been cloned from *B. megaterium* (Curiel-Quesada *et al.*, 1983; Curiel-Quesada and Setlow, 1984; Fliss and Setlow, 1984a,b; Fliss *et al.*, 1985; Hackett *et al.*, 1986) and from *B. subtilis* (Hackett and Setlow, 1987); it is likely that most *Bacillus* species will contain multiple SASPs (Yuan *et al.*, 1981; Johnson and Tipper, 1981; Fliss and Setlow, 1985; Sun and Setlow, 1987). By using a cloned SASP C-3 from *B. megaterium* as a hybridization probe, Fliss and Setlow (1985) were able to demonstrate that the C-3 gene mRNA was made prior to the formation of dipicolinic acid and that at least four SASP genes were expressed in parallel during sporulation.

Several other interesting features of the *B. megaterium* SASP genes have emerged:

(1) The SASP genes are the first extended, divergent, multigene family which has been described in prokaryotes (Fliss *et al.*, 1985).

(2) The upstream regions of the SASP genes have promoters which are similar in sequence in their -35 and -10 regions and may be transcribed by the same RNA polymerase holoenzyme (Fliss *et al.*, 1985). The SASP genes are expressed, however, at different levels.

(3) The ribosome binding sites of the SASP mRNAs are stronger than those usually found in *E. coli* mRNAs (Fliss *et al.*, 1985).

(4) The genes are monocistronic since they all have transcription termination sites just downstream of the translation stop codon. The genes for this multigene family are scattered throughout the chromosome (Fliss *et al.*, 1985).

(5) The DNA sequences of the SASP genes and the amino acid sequence of the SASPs are highly conserved, with about 50% of the amino acid residues conserved and the greatest amino acid changes occurring in the N- and C-terminal regions of the proteins (Fliss *et al.*, 1985). The *B. megaterium* SASPs are related immunologically both among *B. megaterium* SASPs and with SASPs from *B. subtilis* (Connors and Setlow, 1985) and *B. cereus* (Fliss *et al.*, 1985). Thus the genes for these proteins appear to be very highly conserved among different species of *Bacillus*.

(6) The SASPs are in close proximity *in vivo* to the DNA of spores, since they can be crosslinked to DNA by ultraviolet irradiation of whole spores but not of spore extracts (Setlow and Setlow, 1979). It has been proposed that the SASPs may be involved in the resistance of spore DNA to UV light (Setlow, 1975b); since they can bind to DNA *in vitro* (Setlow, 1975a,b), they accumulate in developing spores at the time they become resistant to UV light, and their absence makes spores more sensitive to UV irradiation (Mason and Setlow, 1986, 1987).

Although the functions of the SASPs, beyond that of being storage proteins and possible DNA-binding proteins in the dormant spore, remain obscure, the analysis of the SASP gene family is likely to provide new basic information about the regulation of expression of developmental genes. Since they are expressed in parallel during mid-sporulation, SASP genes provide a model system for studying the regulation of coordinate transcription of widely scattered genes. The conserved regions of the promoters for the SASP genes are not homologous to the previously published consensus promoter sequences (Moran *et al.*, 1982; Doi, 1982a) and may represent promoters recognized by a heretofore unrecognized form of RNA polymerase holoenzyme.

The fact that SASP genes are expressed only in the forespore (Singh *et al.*, 1977) also indicates that differential transcription is occurring in the forespore and

that this is a clear case of compartmentalized transcription and translation in a prokaryotic cell. The question therefore arises as to whether SASP genes have promoters which are recognized by a RNA polymerase occurring only in the forespore? Further investigation of this system should reveal the metabolic signals which control transcription in the forespore, the factors required for specific transcription of genes expressed only in the forespore, the mechanism which determines the relative expression of the SASP genes, and the extent of total gene expression in the forespore relative to that of the mother cell.

A further comparison of the base and amino acid sequence of the SASP genes and their protein products may reveal why some regions of the genes are highly conserved, how these genes have become scattered throughout the genome, what the evolutionary pattern of this gene family is in the various *Bacillus* species, why the genes are monocistronic, and what the functions of SASPs are. Thus for a multiplicity of reasons the SASP family gene system has high potential for providing greater understanding of the sporulation process and the properties of spores.

3.5. Extracellular Enzymes

Another class of proteins which are synthesized after growth has ceased includes the extracellular enzymes (Table II). Gel electrophoresis patterns of extracellular proteins present at T_5 show more than 30 bands (Porter and Mandelstam, 1982). These enzymes are synthesized at the initial stages of sporulation and secreted into the growth medium. Most of the genes for these proteins as well as sporulation genes appear to be repressed by mechanisms similar to catabolite repression, since they are not expressed in the presence of excess glucose or nitrogen sources. The genes for these extracellular proteins are of particular interest for biotechnology (see Chapter 11).

The signal peptides coded by these *Bacillus* extracellular enzyme genes (Kroyer and Chang, 1981; Neugebauer *et al.*, 1981; Palva *et al.*, 1982; Ohmura *et al.*, 1983; Wells *et al.*, 1983; Wong *et al.*, 1984) are similar in composition to signal peptides reported previously in other prokaryotes and in eukaryotes (Kreil, 1981) and have the classic short, charged, N-terminal peptide followed by a long sequence of hydrophobic amino acids and a signal peptidase cleavage sequence (Perlman and Halvorson, 1983; see Chapter 5). The *Bacillus* signal peptides appear on average to be slightly longer than those found in *E. coli*. A number of investigators are attempting to use *B. subtilis* promoters and signal peptides to secrete foreign gene products from *B. subtilis* (see Doi, 1984, for review).

One long-standing question has been whether all enzymatic functions that were derepressed during sporulation had a direct relationship to spore formation. There now appears to be an overall regulatory function which controls the expression of genes for extracellular enzymes and for sporulation. This is best illustrated in the case of the *B. subtilis* extracellular alkaline serine protease (subtilisin) gene (*aprA*). The expression of this gene and the early sporulation genes is controlled by *spo0A* (Hoch and Spizizen, 1969). A mutation in this locus prevents the expression of the subtilisin gene and also blocks sporulation at stage 0. This pleiotropic effect was initially interpreted as an indication that the subtilisin gene was directly associated with sporulation. However, deletion mutations of the subtilisin gene have been obtained recently and these mutant cells are still capable of sporulating as efficiently

Table II. Extracellular Enzymes of *Bacillus* Species

Enzyme and <i>Bacillus</i> species	References ^a
Subtilisin (serine protease)	
<i>B. subtilis</i>	Wong <i>et al.</i> (1984)
	Stahl and Ferrari (1984)
<i>B. amyloliquefaciens</i>	Wells <i>et al.</i> (1983)
	Vasantha <i>et al.</i> (1984a)
Neutral protease	
<i>B. subtilis</i>	Yang <i>et al.</i> (1984)
<i>B. amyloliquefaciens</i>	Vasantha <i>et al.</i> (1984b)
	Shimada <i>et al.</i> (1985)
<i>B. stearothermophilus</i>	Fuji <i>et al.</i> (1983)
Esterase	
<i>B. subtilis</i>	Mamas and Millet (1975)
	Karmazyn-Campelli and Millet (1981)
α -Amylase	
<i>B. subtilis</i>	Yang <i>et al.</i> (1983)
	Yamazaki <i>et al.</i> (1983)
	Shinomiya <i>et al.</i> (1984)
<i>B. amyloliquefaciens</i>	Palva (1982)
	Takkinen <i>et al.</i> (1983)
<i>B. licheniformis</i>	Stephens <i>et al.</i> (1984b)
	Ortlepp <i>et al.</i> (1984)
<i>B. stearothermophilus</i>	Tsukagoshi <i>et al.</i> (1984)
Ribonuclease	
<i>B. subtilis</i>	Nishimura and Nomura (1958)
	Coleman and Elliott (1965)
	Nakai <i>et al.</i> (1965)
Phosphodiesterase	
<i>Bacillus</i> sp.	Jacobsen and Rodwell (1972)
<i>B. subtilis</i>	Nakai <i>et al.</i> (1965)
Deoxyribonuclease	
<i>B. subtilis</i>	Nakai <i>et al.</i> (1965)
	Akrigg and Mandelstam (1978)
Alkaline phosphatase	
<i>B. licheniformis</i>	Hulett (1984)
	Hulett <i>et al.</i> (1985)
<i>B. subtilis</i>	Glenn and Mandelstam (1971)
Sucrase	
<i>B. subtilis</i>	Fouet <i>et al.</i> (1982)
β -Lactamase	
<i>B. cereus</i>	Sloma and Gross (1983)
	Mezes <i>et al.</i> (1983)
Penicillinase	
<i>B. licheniformis</i>	Kroyer and Chang (1981)
	Neugebauer <i>et al.</i> (1981)

^aThese are primarily recent papers concerned with cloning of the genes.

as wild-type cells (Kawamura and Doi, 1984; Yang *et al.*, 1984; Stahl and Ferrari, 1984). Thus although the expression of the subtilisin gene and sporulation genes are under a common control mechanism (*spo0A*), subtilisin is not an essential part of the sporulation process.

These results support the idea that the *spo0A* mutation affects an overall regulatory function which controls the expression of many genes including those for extracellular enzymes and for sporulation, and that these functions are in different pathways and not in a single linear pathway leading to sporulation.

4. METHODS FOR THE ANALYSIS OF SPORULATION AND GERMINATION GENES

With the new recombinant DNA technology (see Chapter 6), several approaches have been developed to identify sporulation and germination loci. This should allow the identification and characterization of the approximately 42 sporulation loci or operons, which were calculated to be present on the *B. subtilis* chromosome by Hranueli *et al.* (1974), and many other germination and outgrowth loci. The complexity of the sporulation and germination process is illustrated by the identification of at least 60 genes and/or loci for sporulation (*spo*), germination (*ger*), and outgrowth (Piggot and Hoch, 1985). A multiplicity of other genes was also demonstrated which have an effect on the initial stages of sporulation (e.g., *rpoD*, *che*); several of these may function during both vegetative growth and sporulation. The exact nature and function of most of the *spo* and *ger* genes are still unknown. Nevertheless rapid progress is being made in cloning these genes and identifying their products, e.g., by homology with known proteins. Thus it would be very useful to have a full catalogue of sporulation and germination genes. Several methods have been developed recently which should facilitate the identification and characterization of these developmental genes.

4.1. Transposon-Directed Insertional Mutagenesis

Streptococcus faecalis transposon Tn917 has been used to create mutations in the *B. subtilis* chromosome; this has resulted in the isolation of various sporulation-defective phenotypes (Youngman *et al.*, 1984a,b; see Chapter 6, Sections 7 and 9).

An analysis of these transposon insertion mutants indicated that a variety of auxotrophs were obtained at a frequency of 5–8%. In addition, *spo* mutants were observed at a frequency of 0.1–0.5% (Youngman *et al.*, 1984b). Of the eight *spo* mutants identified, all eight insertional mutations were distinct from each other and two of the eight were identified as completely new *spo* sites which were not detected by previous selection methods. Thus this method has the potential for isolating many heretofore unidentified *spo* and *ger* loci (Youngman *et al.*, 1985).

Once a transposon insertional mutation has been located in a *spo* locus, it would be extremely useful to be able to isolate relatively quickly all or part of the *spo* locus for further analysis. Isolation of the transcription regulatory region of the locus would allow, for instance, an analysis of the promoter and other putative transcriptional regulatory sites of this developmental locus. The isolation of a portion of the

locus would also permit its use as a hybridization probe of a gene bank of *B. subtilis* and the subsequent identification of a clone which contained the entire locus.

Youngman *et al.* (1984a) developed a novel method for rapid cloning of portions of *B. subtilis* *spo* genes adjacent to Tn917 insertions in the chromosome. The overall rationale for their method is described in Chapter 6, Section 7.4.

This method has been used to isolate a portion of the *spoIIH* gene of *B. subtilis* and can potentially be used to isolate a portion of any gene in which Tn917 resides (Youngman *et al.*, 1984a–c; 1985).

4.2. Use of Gene Fusions for the Study of the Functions of Temporally Regulated and Sporulation Genes

Since the exact functions of most sporulation genes, even those that have been cloned, are still unknown, it is difficult to measure their activities *in vivo* or *in vitro*. Also, if the regulation of these genes is to be analyzed, a method is required to measure the expression of these genes in various genetic backgrounds and under different physiological conditions. If the gene product happens to be a structural protein without enzymatic activity, it becomes extremely difficult to measure directly the regulation of such a gene. One way to overcome this difficulty is to fuse the transcriptional regulatory regions of sporulation genes with a gene whose expression is readily detectable, e.g., an easily assayable enzyme. Then the expression of the sporulation gene can be monitored by assaying the activity of the enzyme. Two such general systems have been reported (Goldfarb *et al.*, 1981; Zuber and Losick, 1983; Youngman *et al.*, 1984b).

4.2.1. Gene Fusions with Tn9-Derived Chloramphenicol Acetyltransferase

In one case a fusion product was formed between the N terminus of a temporally regulated *B. subtilis* gene and the Tn9-derived chloramphenicol acetyltransferase (*cat*) gene present in plasmid pGR71 (Goldfarb *et al.*, 1981). pGR71 is a cointegrate shuttle plasmid which can replicate in either *B. subtilis* or *E. coli*. The gene fusion is made possible by having a promoterless Tn9-derived *cat* gene preceded by a *Hind*III insertion site present in the plasmid. When *Hind*III fragments were shotgun-cloned into this site, insertional activation of the *cat* gene occurred. Most of the products consisted of fusion polypeptides between N-terminal peptides of *B. subtilis* and the C terminus of the *cat* gene product. Since the *cat* gene has an *E. coli* ribosome binding site (RBS) which is not used efficiently by the *B. subtilis* translational machinery, all translation started at the *B. subtilis* RBS on the DNA insert (Goldfarb *et al.*, 1982).

The temporally regulated promoter isolated by this method (Goldfarb *et al.*, 1983a) was found to control the expression of the *B. subtilis* subtilisin gene (*aprA*) (Wong *et al.*, 1984). The translation of the fusion gene began at the ribosome binding site provided by the *aprA* gene and the in-phase translation continued through the leader region of the *cat* gene into the structural portion of the gene. The fusion products were enzymatically and immunologically active (Goldfarb *et al.*, 1982). The response of the gene to different temporal and physiological condi-

tions was measured by analyzing the CAT activity at different times of the growth curve and in the presence or absence of various nutritional factors (Goldfarb *et al.*, 1983a,b). The *cat* gene was expressed only during the stationary phase and with the same pattern as that observed for subtilisin synthesis. Although the *aprA* gene is temporally expressed at a similar time as the *spoII* genes and its expression is controlled by *spoOA* gene, deletion analyses have shown that the *aprA* gene is not required for sporulation (Kawamura and Doi, 1984). Other efficient promoters and fusion products (Wang and Doi, 1984) and the phenomenon of translational coupling have also been analyzed by this fusion system (Zaghloul *et al.*, 1985; Zaghloul and Doi, 1986, 1987). In principle, this system can be applied to the study of most sporulation-specific or sporulation-associated genes.

4.2.2. Fusion of *spoVG* with *E. coli lacZ*

In another type of construction, a system has been devised to study the regulation of the *spoVG* gene by fusing its transcriptional regulatory region to a gene whose product could be measured readily (Zuber and Losick, 1983). Furthermore the system was constructed to allow integration of the fused gene into the chromosome *via* an integrative plasmid (Haldenwang *et al.*, 1980; Ferrari *et al.*, 1983; Price *et al.*, 1983; see Chapter 6) and to study its expression *in vivo* under different regulatory conditions.

For this purpose the N terminus of *spoVG* was fused to the *E. coli lacZ* gene contained in the plasmid pZL207 (Zuber and Losick, 1983). This plasmid contained, in addition to the fusion gene, a *S. aureus* chloramphenicol resistance gene and a pBR322-derived replicon. This plasmid could be used as an integrative plasmid in *B. subtilis* by transforming into this organism and selecting for chloramphenicol resistance. Since no *B. subtilis* origin of replication (*ori*) was present, chloramphenicol resistance could only be expressed by integration of the plasmid into the chromosome by homologous recombination between the *spoVG* gene in the plasmid and in the chromosome. This integration provides a single copy of the regulatory region of *spoVG* fused to the *lacZ* gene.

The expression of the *spoVG* gene, as measured by β -galactosidase synthesis, was normal in a wild-type background. However, its expression was impaired in the presence of mutations in *spoOB*, *spoOC*, *spoOE*, *spoOF*, *spoOH*, *spoJ*, and *spoK* genes. Deletion experiments indicated that the regulation of *spoVG* by the *spoO* genes was exerted at or near the *spoVG* promoter region. Since *spoVG* has a σ^B promoter (see Section 6.1), these results also indicated that many *spoO* functions are required for the expression of genes controlled by σ^B promoters.

The normal expression of the *spoVG* gene was altered considerably if the fusion gene was present on a high-copy-number plasmid; the β -galactosidase activity was expressed considerably earlier in the growth phase and its induction was not impaired by *spoOB* and *spoOH* mutations. A high copy number of *spoVG* promoter also inhibited sporulation (Banner *et al.*, 1983). These results suggest that the amplified *spoVG* promoter may titrate a regulatory protein required early in sporulation and becomes limiting for the expression of *spoVG* and other sporulation genes (Zuber and Losick, 1983).

The use of fusion genes for structural protein genes such as spore coat pro-

teins, membrane proteins, and small acid-soluble spore proteins will allow a much more precise analysis of their expression and regulation during sporulation.

4.2.3. Transposon-Mediated Gene Fusions

As a further improvement of the fusion method, a transposon-mediated gene fusion system has been developed that allows the identification of temporally controlled and sporulation promoters and their genes (Youngman *et al.*, 1984a,b,c; see Chapter 6).

4.3. Cloning of Temporally Controlled Promoters

Although the isolation of a specific gene is most advantageous, there are occasions when the rapid isolation of a number of temporally controlled promoters may be desired. For this purpose a number of promoter expression probe plasmids have been developed for use in *B. subtilis*. These plasmids contain a replication origin (*ori*) for *B. subtilis*, a selectable gene without its promoter preceded by a suitable restriction site, and, often, an antibiotic resistance marker (see Chapter 6).

However, there is a slight complication when temporally regulated promoters are sought, e.g., sporulation or stationary phase promoters. Not all the expression probe plasmids work equally well in this situation. This is particularly true if an antibiotic gene is the selection gene. Since antibiotic resistance will not develop until the stationary phase with temporally regulated promoters, it is not possible to use antibiotic-containing plates for selection of the activated gene. If a selection gene is based on enzyme activity which provides a color and/or morphological signal, then it is possible to look for the late development of these signals relative to the early development of signals by promoters expressed during the exponential phase of growth. Kawamura *et al.* (1986) and Wang and Doi (1987) recently developed a subtilisin expression probe plasmid carried in a protease-deficient strain which develops halos on casein agar plates at about 10 hr with exponential phase promoters and at 20 hr for stationary phase promoters. Thus it is possible to distinguish developmental from exponential phase promoters quite readily with this expression probe plasmid. In addition, it has utility in the isolation of nutritionally controlled promoters, e.g., glucose-sensitive and insensitive promoters.

The use of these expression probe plasmids has led to the isolation of temporally regulated promoters (Goldfarb *et al.*, 1983a; Mongkolsuk *et al.*, 1983), catabolite-repressed and amino acid-controlled promoters (Goldfarb *et al.*, 1983b), as well as a large number of exponential phase promoters with differing degrees of promoter strengths and complexities (Goldfarb *et al.*, 1981).

4.4. Integrative Mapping and Cloning by Gene Conversion Techniques

Useful methods have been developed for mapping *B. subtilis* genes for which no mutations are known or for which assays are difficult, and for the rapid cloning of mutant genes when the wild-type gene has been cloned.

4.4.1. Integrative Mapping

For the mapping of a cloned *B. subtilis* gene in which no mutations are available for complementation studies, the rationale is to insert the gene into an *E. coli* plasmid containing an antibiotic resistance marker which can be expressed in *B. subtilis* and then transforming the plasmid into *B. subtilis*. Since no origin of replication (*ori*) exists for *B. subtilis*, the only antibiotic-resistant clones which will appear are those in which the plasmid has been integrated into the host chromosome by homologous recombination between the gene of interest in the plasmid and in the host chromosome. The antibiotic resistance marker is inserted close to the gene of interest in the *B. subtilis* chromosome and is then mapped by PBS1 transduction analysis using the set of mapping mutants developed by Dedonder *et al.* (1977) (see Chapter 4). This integrative mapping technique was first demonstrated for mapping of the 0.4-kilobase *spoVG* gene in *B. subtilis* by Haldenwang *et al.* (1980).

As another example of this type of analysis, the σ^{43} (σ^A) gene (*rpoD*) was cloned (Price *et al.*, 1983) by immunoscreening a *B. subtilis* gene bank constructed in λ gt WES (Ferrari *et al.*, 1981). Since no *rpoD* mutants were known at this time, integrative mapping was used to find the locus of the *rpoD* gene. The cloned *rpoD* was inserted into integrative plasmid pCP112 (Price *et al.*, 1983) which contained an *E. coli ori* from plasmid pBR327 (Soberon *et al.*, 1980) and the chloramphenicol resistance (*Cm^r*) gene from PC194 (Horinouchi and Weisblum, 1982). This plasmid was transformed into *B. subtilis* and clones were selected for *Cm^r*. By transduction analyses using *Cm^r* as the selection marker, the location of *rpoD* was found to be at around 225° on the circular *B. subtilis* map (Price *et al.*, 1983; Price and Doi, 1985).

4.4.2. Gene Conversion

There are occasions when it is useful to clone several DNA fragments containing mutations at various locations in the same gene, e.g., to do sequence analyses of the DNAs to find the precise locations of the mutations. A rapid method has been developed for achieving this, based on gene conversion which is carried out actively by *B. subtilis*, presumably by its efficient mismatch repair mechanism (Chak *et al.*, 1982; Iglesias and Trautner, 1983; Kawamura and Doi, 1984).

This method depends initially on cloning a gene of interest on a *B. subtilis* plasmid such as pUB110. When a recombinant plasmid containing the wild-type gene is transformed into a host containing a chromosomal mutation of the gene, three types of clones will result. About 90–95% of the clones will represent the original transformant type in which the wild-type gene will be on the plasmid and the mutant gene will be in the chromosome; no gene conversion has occurred in this type. In the second type the clone will contain the wild-type gene in both the plasmid and in the chromosome; in this case the wild-type gene has replaced the mutant gene in the chromosome by gene conversion (we refer to this as “beaming down” of the wild-type gene from the plasmid to the chromosome). In the third type the clone will contain the mutant gene in both the plasmid and in the chromosome. In this case the mutant gene of the chromosome has replaced the wild-type gene in the plasmid by gene conversion (we refer to this as “beaming up” of the mutant gene from the chromosome to the plasmid).

The utility of the system is now evident, since if one selects for a mutant phenotype, one can find the mutant gene in the plasmid and the gene can then be isolated by plasmid screening for further analysis. However, the gene of interest has to be cloned first and be present on the plasmid before this technique is applicable. As an example of this type of analysis, the *crsA* mutations in the *rpoD* gene were beamed up from the chromosome of *B. subtilis* to plasmids and the exact locations of the *crsA* mutations within the *rpoD* gene were determined by base sequence analysis of the mutant genes residing in the plasmid (Kawamura *et al.*, 1985).

5. ISOLATION AND CHARACTERISTICS OF CLONED SPORULATION AND GERMINATION GENES

Over 50 genes and loci for sporulation and germination have been identified (Piggot and Hoch, 1985; Errington *et al.*, 1985). The characterization of these genes is essential for elucidating the regulatory and structural components of the sporulating cells. For this purpose a significant number of sporulation and germination genes have been cloned. Most of the cloning procedures have been based on finding DNA fragments which complement the Spo⁻ phenotype. The cloned fragments have usually been carried on plasmids or temperate phage of *B. subtilis* (Iijima *et al.*, 1980; Kawamura *et al.*, 1980a,b; Kawamura *et al.*, 1981). A list of cloned *spo* and *ger* genes is presented in Table III. Although the exact functions of all but two of these genes are still unknown, the probable function of some of the genes has been identified by comparing the amino acid sequence homology of their gene products with that of known proteins.

The presence of some of the *spo* genes on high-copy plasmids inhibits sporulation. Recombinant plasmids carrying *spo0F* (Kawamura *et al.*, 1980a), *spoIIG* (Ayaki and Kobayashi, 1984), *spoIVC* (Fujita and Kobayashi, 1985), *spoVE* Yanada *et al.*, 1983), and *spoVG* (Banner *et al.*, 1983) inhibit sporulation of the wild-type strain, whereas those carrying *spo0B* (Hirochika *et al.*, 1981) do not have any effect on sporulation. Thus it is possible that these genes could titrate limiting cellular components (minor RNA polymerase holoenzymes or regulatory proteins) or produce proteins which are detrimental to sporulation at higher concentrations.

5.1. *spo0* Genes

Of the 10 known *spo0* loci (Piggot and Hoch, 1985) *spo0A*, *spo0B*, *spo0F*, and *spo0H* have been cloned and sequenced. Most of the *spo0* loci appear to code for one protein.

5.1.1. *spo0A*

In the hierarchy of *spo0* mutations, *spo0A* exerts the greatest pleiotropic effect and is believed to be of major importance (Piggot and Coote, 1976). The gene has been cloned (Ikeuchi *et al.*, 1983; Ferrari *et al.*, 1984) and codes for a protein with a molecular mass of 27,500 according to Kudoh *et al.* (1984) or 29,691 according to

Table III. Cloned Sporulation and Germination Genes

Gene	Molecular masses of product	References
<i>spo0A</i>	29,691	Ferrari <i>et al.</i> (1985b) Kudoh <i>et al.</i> (1985) Ikeuchi <i>et al.</i> (1983)
<i>spo0B</i>	22,542	Bouvier <i>et al.</i> (1984) Bonamy and Szulmajster (1982) Ferrari <i>et al.</i> (1982) Hirochika <i>et al.</i> (1981) Ferrari <i>et al.</i> (1985a)
<i>spo0C^a</i>		Ikeuchi <i>et al.</i> (1983) Ferrari <i>et al.</i> (1985b)
<i>spo0F</i>	14,286	Trach <i>et al.</i> (1985)
<i>spo0H</i> (<i>B. subtilis</i>)	30,000	Weir <i>et al.</i> (1984) Dubnau <i>et al.</i> (1987)
<i>spo0H</i> (<i>B. licheniformis</i>)	22,000	Ramakrishna <i>et al.</i> (1984) Dubnau <i>et al.</i> (1987)
<i>spoIIA</i>		Savva and Mandelstam (1984)
<i>spoIIAA</i>	13,100	Fort and Piggot (1984)
<i>spoIIAB</i>	16,300	Fort and Piggot (1984)
<i>spoIIAC</i>	22,200	Fort and Piggot (1984)
<i>spoIIC</i>		Anaguchi <i>et al.</i> (1984)
<i>spoIIG</i>	27,652	Stragier <i>et al.</i> (1984) Trempy <i>et al.</i> (1985b) Ayaki and Kobayashi (1984)
<i>spoIIIB</i>		Jenkinson and Mandelstam (1983)
<i>spoIIIE</i>		Butler and Mandelstam (1987)
<i>spoIVC</i>		Fujita and Kobayashi (1985)
<i>spoVAA</i>	23,100	Fort and Errington (1985)
<i>spoVAB</i>	15,200	Fort and Errington (1985)
<i>spoVAC</i>	16,100	Fort and Errington (1985)
<i>spoVAD</i>	36,000	Fort and Errington (1985)
<i>spoVAE</i>	34,300	Fort and Errington (1985)
<i>spoVE</i>		Yamada <i>et al.</i> (1983) Piggot <i>et al.</i> (1986)
<i>spoVG</i>		Segall and Losick (1977)
SASP I (<i>B. subtilis</i>)		Connors and Setlow (1985)
SASP C (<i>B. megaterium</i>)		Curiel-Quesada <i>et al.</i> (1983) Fliss and Setlow (1984a,b)
SASP C-1-C-5 (<i>B. megaterium</i>)		Fliss <i>et al.</i> (1985)
<i>gerA</i>		Moir (1981) Zuberi <i>et al.</i> (1987)
<i>gerD</i>		Moir <i>et al.</i> (1985)
<i>gerE</i>	8,500	Cutting and Mandelstam (1986)
<i>gerJ</i>		Warburg <i>et al.</i> (1985)
<i>gerM</i>		Moir <i>et al.</i> (1985)
<i>gdh</i>	31,000	Vasantha <i>et al.</i> (1983)

^a*spo0C* is now known to be a locus within *spo0A* (Ferrari *et al.*, 1985b).

Ferrari *et al.* (1985b). Genetic experiments have indicated that *spo0C* is actually a mutation in the C terminus of the *spo0A* gene (Ferrari *et al.*, 1985b) and that the mutations *sof* (Kawamura and Saito, 1983) and *rvt* (Sharrock *et al.*, 1984) are mutations in the N terminus of the *spo0A* gene (Hoch *et al.*, 1985). Although most mutations in the *spo0A* locus cause asporogeny, other mutations in the locus such as *sof* and *rvt*, which are probably identical mutations (Hoch *et al.*, 1985), allow the cell to sporulate. These mutations affect the N-terminal domain of the *spo0A* gene product, which still allows it to function and to suppress other *spo0* functions. Mutations in other domains of the *spo0A* gene product, however, do not allow it to function for sporulation. Therefore the Spo0A phenotype depends on the site of mutation in the *spo0A* gene and the actual function of the *spo0A* is probably not specific for sporulation. The Spo0A phenotype may arise as a consequence of the general function of the *spo0A* gene product.

The postulated major role for *spo0A* in the initiation of sporulation is supported by its ability to suppress other *spo0* mutations, including even a deletion mutation of *spo0F* (Kawamura and Saito, 1983; Sharrock *et al.*, 1984). An altered Spo0A protein (Sof or Rvt protein) can overcome the requirements for the *spo0F*, *spo0E*, and *spo0B* gene products. These results suggest that the products of the *spo0B*, *spo0E*, and *spo0F* genes normally modulate the function of the *spo0A* product; however, this modulation is unnecessary when a *sof* or *rvt* mutation is present which allows the mutant *spo0A* product to bypass the *spo0B*, *spo0E*, and *spo0F* functions (Hoch *et al.*, 1985).

The *spo0A* gene is expressed primarily during the exponential phase of growth and is not essential for growth (Ferrari *et al.*, 1985b). However, its function appears to be absolutely necessary for the initiation of sporulation. A comparison of amino acid sequences has revealed a partial homology between the Spo0A protein with the Spo0F protein, and the OmpR and SfrA products of *E. coli* (Trach *et al.*, 1985). The *ompR* gene product is a regulator of the expression of the *ompC* and *ompF* genes (Hall and Silhavy, 1981a), which code for outer membrane porin proteins of *E. coli*. The *ompR* gene product is a DNA-binding protein and may be a positive regulator of gene expression (Hall and Silhavy, 1981b).

Thus the Spo0A product may also regulate the expression of genes which affect membrane functions in *B. subtilis*. It has been postulated that the *spo0* genes are involved in some type of nutritional sensing of the environment (Ferrari *et al.*, 1985a; Doi *et al.*, 1985). This idea is supported by the finding of Doi *et al.* (1985) that the *sof* mutant also has a Crs (catabolite-resistant sporulation) phenotype (Takahashi, 1979). Thus *spo0A* may code for a regulatory protein which responds to some mechanism or part of an apparatus of the membrane that senses the availability of carbohydrates. The interaction between the Spo0A protein and the sensing device is altered in *sof* mutants and allows sporulation initiation even in the presence of excess glucose (Doi *et al.*, 1985).

5.1.2. *spo0B*

The *spo0B* gene has been cloned by a number of investigators (Hirochika *et al.*, 1981; Bonamy and Szulmajster, 1982; Ferrari *et al.*, 1982; Bouvier *et al.*, 1984) and codes for a protein with a molecular mass of about 22,500. The sequence analysis of

the *spo0B* locus indicates that *spo0B* is part of an operon which contains at least one more downstream gene (Ferrari *et al.*, 1985a).

5.1.3. *spo0C*

The *spo0C* gene has now been identified as a locus in the *spo0A* gene (Ferrari *et al.*, 1985b). The *spo0C* mutation is located in the tenth codon from the C terminus of the *spo0A* gene and gives rise to a missense Spo0A protein which is partially active and results in a phenotype which is less pleiotropic than mutations in other sites of the gene. The *spo0C* gene was cloned along with *spo0A* in a temperate bacteriophage of *B. subtilis* (Ikeuchi *et al.*, 1983).

5.1.4. *spo0F*

The *spo0F* gene codes for a protein of 14,286 Da (Trach *et al.*, 1985, 1986). The derived amino acid sequence of the Spo0F protein shows homology with the N-terminal half of the protein coded by the *spo0A* gene and the OmpR and SfrA proteins of *E. coli* (Trach *et al.*, 1985). Since *ompR* controls the expression of *ompC* and *ompF* genes (see 5.1.1), *spo0F* like *spo0A* may regulate expression of genes which code for *B. subtilis* membrane components. Since membranes are involved in the sporulation process, the Spo phenotype of mutations in these genes may be an indirect consequence of damage to this regulatory system (Trach *et al.*, 1985).

The gene cloned initially by Shimotsu *et al.* (1983) as *spo0F* codes for a protein of 19,065 Da. This gene has now been located adjacent to the *spo0F* gene (Trach *et al.*, 1986; Yoshikawa *et al.*, 1986a,b). The *spo0F221* mutation (Yoshikawa *et al.*, 1986b) has been located to a codon for a leucine residue in the N terminus of the *spo0F* gene (Trach *et al.*, 1985).

The *spo0F* mutation can be suppressed by *sof* (suppressor of *spo0F*), which is actually a *spo0A* mutation (Kawamura and Saito, 1983; Sharrock *et al.*, 1984). Since even a deletion mutant of *spo0F* can be suppressed by *sof*, it indicates that the Spo0F protein normally modulates the activity of the Spo0A protein and that the latter plays a more direct role than the former in initiating sporulation.

5.1.5. *spo0H*

The *spo0H* gene has been cloned from *B. subtilis* (Weir *et al.*, 1984) and *B. licheniformis* (Ramakrishna *et al.*, 1984). The *B. subtilis* gene codes for a protein with a molecular mass of 30,000. Spo0H has amino acid homology with the major RNA polymerase σ^{43} factor and has been shown to be a minor σ factor (Dubnau *et al.*, 1987; Carter and Moran, 1987). This σ factor has been designated as σ^H and its gene as *sigH*.

5.1.6. *spo0K*

Although the *spo0K* gene has not been cloned to date, the mutual suppression between *rpoD47* (*crsA47*) and *spo0K* suggests that it may be a transcriptional regulatory factor (Kawamura *et al.*, 1985).

5.2. *spoII* Genes

Seven *spoII* loci control the developmental stages after forespore septum formation (Piggot and Hoch, 1985). The *spoIIA* (Savva and Mandelstam, 1984; Fort and Piggot, 1984), *spoIIC* (Anaguchi *et al.*, 1984), and *spoIIG* (Bonamy and Szulmajster, 1982; Ayaki and Kobayashi, 1984) genes have been cloned and sequenced.

It was first noted by sequence analyses that the *spoIIG* gene product had a molecular mass of about 28,000 and a partial amino acid sequence homology with the *E. coli* σ^{70} factor (Stragier *et al.*, 1984) and the *B. subtilis* σ^{43} factor (Gitt *et al.*, 1985). Subsequent genetic studies by Trempy *et al.* (1985b) showed that *spoIIG* codes for the sporulation-specific σ^E factor (Trempy *et al.*, 1985c) of *B. subtilis* RNA polymerase (Haldenwang *et al.*, 1981). The σ^E factor is the first sporulation-specific σ factor which has been identified and mapped genetically. The protein is synthesized initially as a precursor with a molecular mass of 31,000 (Trempy *et al.*, 1985a; LaBell *et al.*, 1987) in the mother cell (Fukuda and Doi, 1977) and then is processed and transported to the forespore where it is found as a mature 29,000-Da protein (Nakayama *et al.*, 1980). It is possible that most genes controlled by σ^E promoters will be expressed in the forespore.

The recent sequence analysis of the *spoIIA* locus indicated that three genes were present in this site, coding for proteins with molecular masses of 13,100, 16,300, and 29,000 (Fort and Piggot, 1984). The protein product with a molecular mass of about 29,000 also has partial amino acid sequence homology (Errington *et al.*, 1985) with σ^{43} factor of *B. subtilis* (Gitt *et al.*, 1985). Thus it too is potentially a sporulation-specific factor, although more biochemical and genetic evidence is required for substantiation of this hypothesis.

The synthesis of sporulation-specific σ factors at stage II indicates that the RNA polymerase at this point is able to transcribe a number of sporulation genes, although no products have been identified as yet from σ^E -controlled genes. It is possible that sporulation-associated genes are also controlled by σ^E , since several intracellular and extracellular enzymes are synthesized at this time.

5.3. *spoIII* Genes

The *spoIII* genes control development of the forespore after engulfment has occurred. Six loci have been reported for these genes (Piggot and Hoch, 1985). The *spoIIIB* gene has been cloned in phage $\phi 105$ (Jenkinson and Mandelstam, 1983).

In an approach to determine whether a gene was expressed in the mother cell or the forespore, transformation of *spo* mutants which were blocked at or after stage III was examined (DeLencastre and Piggot, 1979). If transformation of *Spo*⁻ strains yielded *Spo*⁻ spores, then it was assumed that the site for expression of the *spo* gene was on the mother cell chromosome, i.e., the genome of the mother cell during sporulation had been transformed to *Spo*⁺, but not that of the forespore. Also it was assumed that transformation of only the mother cell genome was sufficient for sporulation to occur. Since the mother cell is destroyed upon release of the mature spore, the nontransformed spore genome would still be *Spo*⁻.

From these studies it was concluded that *spoIIID*, *spoIVA*, *spoVB*, and *spoVE* were expressed in the mother cell. For one locus, *spoVA*, it was assumed that it had

to be expressed in the forespore, since greater than 99% of the resulting spores were Spo^+ .

5.4. *spoIV* Genes

The seven *spoIV* loci are expressed during cortex formation. The *spoIVC* gene has been cloned by the prophage transformation method using phage $\phi 105$ (Fujita and Kobayashi, 1985). The *spoIVC* (Dancer and Mandelstam, 1981) and *spoIVF* (Lamont, 1984) loci both contain at least two genes.

Although $SpoIV^-$ cells develop normally until stage IV, an analysis of their protein synthesis patterns reveals that they differ from the wild type as early as T_2 (i.e., stage II) (Boschwitz and Yudkin, 1983). Thus these and other (Jenkinson *et al.*, 1981) results cast some doubt on the classification of mutants, since it might be assumed that stage IV mutants follow a normal pattern of development up to stage IV.

By transformation experiments described in Section 5.3, it was determined that *spoIVA* was expressed from the mother cell genome rather than the forespore genome (DeLencastre and Piggot, 1979).

5.5. *spoV* Genes

Many of the nine *spoV* loci are involved in the assembly of the spore coat proteins on the forespore. The *spoVA* locus has been sequenced and it contains five open reading frames coding for proteins of molecular masses of 15,000–36,000 (Fort and Errington, 1985). It is the largest polycistronic sporulation operon yet characterized. Although the functions of these *SpoV* proteins are not known, they are highly basic and hydrophobic, which suggests that they may have some relationship to SASP, membrane, or DNA-binding proteins.

spoVA is expressed in the forespore whereas *spoVB* is expressed in the mother cell (DeLencastre and Piggot, 1979). *spoVG*, previously called the 0.4-kilobase gene (Haldenwang *et al.*, (1980), maps near *tms26* at 6° (Piggot and Hoch, 1985).

The interpretation of *spoV* mutants is complicated by the fact that the formation of the spore coat in stage V involves the deposition of coat proteins which had been synthesized during stages II and III (Jenkinson *et al.*, 1981). Also the *spoVG* gene is transcribed within 30 min of the start of sporulation (Segall and Losick, 1977). Thus it is highly likely that mutations in genes expressed early in sporulation are resulting in a stage V phenotype.

5.6. *spoVI* Genes

The two *spoVI* loci are involved in the maturation of the spore. The spores produced by *spoVIA* mutants germinate slowly and are sensitive to lysozyme (Jenkinson, 1981). Their spore coats lack a 36,000-Da protein which is normally deposited during stage V. This locus is different from the *ger-36* mutant which maps close by (Moir *et al.*, 1979).

Another locus, *spoVIB*, is involved in the proper assembly of coat proteins

(Jenkinson, 1983). The assembly of the coat is delayed in *spoVIB* mutants and misarrangement of the surface proteins, particularly a 12,000-Da protein, is evident. These spores are very sensitive to lysozyme and sporulate and germinate more slowly than wild-type cells.

5.7. *ger* Genes

Mutations in the 13 *ger* loci cause defective germination of spores in a range of germinants (Moir *et al.*, 1985). More than 100 mutants of *B. subtilis* have been isolated whose spores germinate abnormally. Although several of the *ger* genes have been cloned, they have not as yet been thoroughly characterized.

5.7.1. *gerA* Genes

The *gerA* mutants are altered in their response to alanine and related amino acid germinants, but germinated normally in a mixture of glucose, fructose, asparagine, and KCl (Moir *et al.*, 1985). The *gerA* locus, which maps close to *citG*, may encode a germination receptor common to L-alanine and its analogs. Three *gerA* complementation groups have been identified (Zuberi *et al.*, 1985), and sequence studies revealed the presence of three genes that code for polypeptides with masses of 53,506, 41,257, and 42,363 Da (Zuberi *et al.*, 1987).

5.7.2. *gerD* Gene

The *gerD* gene is located close to *rpoB* and *rpsE* (Moir *et al.*, 1979). Germination in L-alanine or in a mixture of L-alanine, glucose, fructose, and KCl was slower than that of the wild type. Plasmids carrying DNA from the *gerD* region have been isolated (Moir *et al.*, 1985).

5.7.3. *gerE* Gene

The *gerE* gene maps near *uvrB* and *spoVIA*. The altered germination of these spores may be due to an alteration in a spore coat protein (Moir, 1981). The *gerE* region has been cloned and sequenced and codes for a small protein of 74 amino acid residues (Cutting and Mandelstam, 1986).

5.7.4. *gerJ* Gene

The *gerJ* locus is located close to *aroC* and *ser-22*. *gerJ* mutants form phase bright spores, which respond to the same germinants as wild type, but during germination they reach only a phase grey instead of a phase dark stage. Their spores also acquire resistance to heat and organic chemicals more slowly than the wild type and the spores are more sensitive to heating at 90°C. Their spores may have an altered cortex structure perhaps caused by a defect in an enzyme involved in cortex synthesis (Warburg *et al.*, 1985).

5.7.5. *gerM* Gene

The *gerM* is linked to *citF* and *ilvB*. The *gerM96* mutant forms lysozyme-resistant spores which germinate only to an intermediate stage in L-alanine or in a mixture of glucose, fructose, L-asparagine, and KCl. The loss of heat resistance was normal, but the germinating spores only achieved a phase grey state. Plasmids carrying the *gerM96* gene were isolated (Moir *et al.*, 1985) by the rescue vector method of Youngman *et al.* (1985) (see Section 4.1 and Chapter 6, Section 7.4).

6. TRANSCRIPTIONAL REGULATION DURING SPORULATION

Transcriptional regulation during sporulation has been demonstrated by the appearance of new classes of mRNA (Doi and Igarashi, 1964; Yamakawa and Doi, 1971; Sumida-Yasumoto and Doi, 1977) and of sporulation-associated and sporulation-specific proteins (see Section 3). This differential expression of sporulation genes is controlled by a complex transcription mechanism consisting of an array of RNA polymerase σ factors (Losick and Pero, 1981; Doi, 1982a,b; Doi and Wang, 1986), each allowing the temporal recognition of different sets of promoters (Moran *et al.*, 1981a,b, 1982; Johnson *et al.*, 1983), and the activity of other transcription regulatory factors which have not been characterized to date.

6.1. Multiple RNA Polymerase Holoenzymes

The presence of multiple RNA polymerase holoenzymes in *B. subtilis* has been well documented. In the vegetative cell there are at least four RNA polymerase holoenzymes which differ by virtue of having different σ factors associated with a common core enzyme (Table IV). The major form of the enzyme is designated as $E\sigma^{43}$ or $E\sigma^A$ (E = core enzyme; the number indicates the molecular mass of the σ factor $\times 10^{-3}$; the superscript is also used to describe a particular σ factor). This enzyme form was previously designated as $E\sigma^{55}$ but recent sequence analysis of the major σ factor gene showed that the molecular mass was closer to 43,000 (Gitt *et al.*, 1985). $E\sigma^{43}$ comprises about 90–95% of the RNA polymerase present in vegetative cells (Doi *et al.*, 1980). The minor forms of vegetative RNA polymerase contain σ factors with molecular masses of 37,000 (σ^{37} or σ^B) (Haldenwang and Losick, 1980), 32,000 (σ^{32} or σ^C) (Johnson *et al.*, 1983), 30,000 (σ^{31}) (Dubnau *et al.*, 1987; Carter and Moran, 1987), and 28,000 (σ^{28} or σ^D) (Wiggs *et al.*, 1981), and these forms compose about 5–10% of the total enzyme. The promoter specificities of these holoenzymes are listed in Table V.

$E\sigma^B$ is most active near the end of the exponential phase of growth (Losick, 1982). The *sigB* gene has been cloned and sequenced (Binnie *et al.*, 1986; Duncan *et al.*, 1987). $E\sigma^D$ appears to have an activity pattern similar to that of $E\sigma^B$. $E\sigma^D$ is active during growth, but its activity drops severely at the end of exponential growth (Gilman and Chamberlin, 1983).

$E\sigma^{43}$ plays a major role in transcription during growth, but its activity during sporulation is still not certain (Linn *et al.*, 1973). Although it can be found in sporulating cells (Tjian and Losick, 1974; Fukuda and Doi, 1977), it has not been

Table IV. RNA Polymerase Holoenzyme Forms in *B. subtilis*

Stage of Growth	RNA polymerase	References
Vegetative	$E\sigma^{43a}$	Shorenstein and Losick (1973)
	($E\sigma^A$)	Fukuda <i>et al.</i> (1975)
		Gitt <i>et al.</i> (1985)
	$E\sigma^B$	Haldenwang and Losick (1980)
		Duncan <i>et al.</i> (1987)
Stage 0		Binnie <i>et al.</i> (1986)
		Igo <i>et al.</i> (1987)
	$E\sigma^C$	Johnson <i>et al.</i> (1983)
	$E\sigma^D$	Wiggs <i>et al.</i> (1981)
	$E\sigma^{43}$	Tjian and Losick (1974)
Stage II		Doi <i>et al.</i> (1983)
	$E\sigma^B$	Haldenwang and Losick (1980)
		Johnson <i>et al.</i> (1983)
	$E\sigma^D$	Gilman and Chamberlin (1983)
	$E\sigma^{43}$	Tjian and Losick (1974)
		Doi <i>et al.</i> (1980)
	$E\sigma^B$	Johnson <i>et al.</i> (1983)
	$E\sigma^C$	Johnson <i>et al.</i> (1983)
	$E\sigma^E$	Fukuda <i>et al.</i> (1975)
		Linn <i>et al.</i> (1975)
Stage III		Fukuda and Doi (1977)
		Nakayama <i>et al.</i> (1978)
		Haldenwang <i>et al.</i> (1981)
		Stragier <i>et al.</i> (1984)
		Trempey <i>et al.</i> (1985bc)
	$E\sigma^D$	Gilman and Chamberlin (1983)
	$E\sigma^{Fb}$	Fort and Piggot (1984)
		Errington <i>et al.</i> (1985)
	$E\sigma^{43}$	Tjian and Losick (1974)
		Doi <i>et al.</i> (1980)
	$E\sigma^B$	Johnson <i>et al.</i> (1983)
	$E\sigma^C$	Johnson <i>et al.</i> (1983)
	$E\sigma^E$	Fukuda <i>et al.</i> (1975)
		Linn <i>et al.</i> (1975)
		Nakayama <i>et al.</i> (1978)
		Haldenwang <i>et al.</i> (1981)
		Stragier <i>et al.</i> (1984)
		Trempey <i>et al.</i> (1985bc)
	$E\sigma^{Fb}$	Fort and Piggot (1984)
		Errington <i>et al.</i> (1985)

^a $E\sigma^{43}$ ($E\sigma^A$) was previously designated as $E\sigma^{55}$ based on its electrophoretic mobility. The sequence analysis indicates that σ^A has a molecular weight of around 43,000 (Gitt *et al.*, 1985).

^bTentatively identified as a σ factor by sequence similarity to σ^{4F3} (Errington *et al.*, 1985).

Table V. Promoter Sequences for Various Holoenzymes^a

Holoenzyme	-35		-10	References
<i>B. subtilis</i>				
E σ^A (E σ^{43})	TTGACA	N ₁₇	TATAAT	Moran <i>et al.</i> (1982)
E σ^B	N.D.		N.D.	
E σ^C	N.D.		N.D.	
E σ^D	CTAAA	N ₁₆	CCGATAT	Gilman <i>et al.</i> (1981)
E σ^E	GAANAANT		CATATTNT	Rather <i>et al.</i> (1986)
E σ^H	GCAGGANTT		GAATTNNT	Carter <i>et al.</i> (1988)

^aN, any base; N.D., not determined.

demonstrated to function actively during spore formation. The subtilisin gene (*aprA*) appears to be regulated by a σ^{43} promoter which can be utilized as late as T₅ (Park and Doi, unpublished results). Thus σ^{43} enzyme may be active even during late sporulation.

The E σ^D may recognize heat shock protein genes in *B. subtilis*, since a σ^D promoter is found to precede the *rpoD* gene in the σ^{43} operon (Wang *et al.*, 1985; Wang and Doi, 1986a) and the *rpoD* gene responds to heat shock treatment (Wang and Doi, 1986b). However, not all heat shock promoters are σ^D promoters (Gilman and Chamberlin, 1983), since the heat shock response still occurred in *spo0* mutants which no longer made transcripts from σ^D -controlled promoters (Kawamura and Doi, unpublished data). Recent data indicate that E σ^D controls the expression of flagella and chemotaxis genes (Helmann and Chamberlin, 1987).

Since the minor RNA polymerase enzymes found in vegetative cells have been found to be active under stress, e.g., nutritional deprivation and heat shock, and the minor σ enzyme (σ^{32}) in *E. coli* (Grossman *et al.*, 1984) also responds to heat shock situations, it appears that minor enzymes may have evolved to control the expression of stress-related genes of the cell. These could include genes which respond to nutritional deprivation, heat shock, ultraviolet light irradiation, toxic chemicals, genetic invasion by DNA or phage genomes.

6.2. Temporally Regulated Promoters

Two types of temporally regulated promoters have been found to date; these control the expression of genes after the end of the logarithmic phase of growth. One type controls the expression of genes which are not essential for sporulation and the other presumably controls genes essential for normal sporulation.

The *spoVG* and *ctc* genes contain overlapping σ^B and σ^C promoters (Johnson *et al.*, 1983; Stephens *et al.*, 1984a). The *spoVG* is required for normal development of the spore coat and thus it is a sporulation-specific gene controlled in a temporal fashion. The interesting point about this gene is that it is expressed very early in sporulation, at about T_{0.5}, but it regulates the morphological development of the spore at about T₅ (Segall and Losick, 1977). Other temporally regulated promoters have been isolated including σ^B and σ^E promoters (Haldenwang *et al.*, 1981; Wang and Doi, 1984).

Why do many *B. subtilis* genes have more than one promoter? *Bacillus subtilis* is transcriptionally active during growth and the sporulation phase. The analyses of mRNA populations revealed that most of the genes expressed during growth were still expressed during sporulation (Sumida-Yasumoto and Doi, 1977). Since σ^{43} enzyme is utilized during growth and minor σ enzymes are more active during sporulation, it is possible that multiple promoters have evolved so that housekeeping genes are capable of being expressed at all stages of growth.

The presence of multiple promoters for sporulation genes suggests that these genes are expressed over extended periods of the sporulation phase and therefore require promoters which might be expressed during early and late sporulation. Presumably those genes expressed only at late sporulation stages may have only one promoter. Analyses of more promoter regions should reveal whether this is the case.

6.3. σ^E Enzyme

The product of *spoIIIG* gene, σ^E , is synthesized during early sporulation. The sporulation-specific $E\sigma^E$ is necessary for transcription of developmental genes after forespore septum formation. σ^E is one of only two known *spo* gene products (the other being *spo0H* product or SigH) which have been identified to date (presumably SASPs and spore coat proteins are also *spo* genes). σ^E has partial amino acid sequence homology with σ^{43} (Stragier *et al.*, 1984; Gitt *et al.*, 1985).

One of the products of the *spoIIA* locus has a molecular mass of 29,000 (P29) (Fort and Piggot, 1984) and also has partial amino acid sequence homology (Errington *et al.*, 1985) with σ^{43} (Gitt *et al.*, 1985). Thus the occurrence of sporulation σ factors indicates that a sequential expression of σ factors partially controls the temporally regulated expression of sporulation genes as suggested by Losick and Pero (1981). This is supported by the fact that each σ factor controls the recognition of specific promoters (Losick and Pero, 1981; Doi, 1982a; Doi and Wang, 1986). Thus each σ factor regulates the expression of a set of genes containing similar promoter sequences. It is likely that other σ factors will be found for the sporulation phase.

6.4. RNA Polymerase, *spo0* Genes, and Catabolite Repression

A relationship between RNA polymerase activity, *spo0* gene products, and catabolite repression has been observed. The activity of $E\sigma^D$ is regulated by the products from *spo0A*, *spo0B*, *spo0E*, and *spo0F* genes (Gilman and Chamberlin, 1983). The activity of $E\sigma^B$ is regulated by the products from *spo0A*, *spo0B*, *spo0E*, *spo0F*, and *spo0H* genes (Ollington *et al.*, 1981). These results indicate that the products of *spo0* genes are involved either directly or indirectly in transcription by these minor RNA polymerase forms both during growth (σ^D) and during early sporulation (σ^B).

This relationship has been extended even further by the observation that a mutation, *rpoD47* (Price and Doi, 1985; Kawamura *et al.*, 1985), in the major σ^{43} gene (Price *et al.*, 1983) can suppress mutations in *spo0B*, *spo0D*, *spo0F*, *spo0J*, and *spo0K*, but not *spo0A* (Doi *et al.*, 1985; Kawamura *et al.*, 1985; Leung *et al.*, 1985).

The *rpoD47* mutation, initially selected as *crsA47* (catabolite-resistant sporulation) by Takahashi (1979), allows *B. subtilis* cells to sporulate in the presence of high levels of glucose. This and other *crs* mutants were mapped to six loci (*crsA* to *crsF*) on the *B. subtilis* map. The *crsE* locus is within the RNA polymerase *rpoB-rpoC* ($\beta\beta'$) region (Sun and Takahashi, 1984) and the *crsC* locus was mapped close to *spo0A* (Sun and Takahashi, 1982). It appears that *crsC* actually is a mutation in *spo0A* (F. Kawamura, personal communication). This observation is supported by the fact that *sof*, which is a mutation in *spo0A* (Hoch *et al.*, 1985), also displays a Crs phenotype (Doi *et al.*, 1985). It is therefore possible that *crsC* is identical to *sof* or is a mutation very close to it in *spo0A*. Thus mutations in either *rpoD* or *spo0A* result in a catabolite-resistant sporulation. Furthermore *spo0A* can suppress *rpoD47* (*crsA47*) (Kawamura *et al.*, 1985).

Molecular mapping experiments localized the *rpoD47* mutation to a proline codon (CCT) which had been changed to a phenylalanine codon (TTT) at amino acid residue 290 in the σ^{43} gene (*rpoD*) (Price and Doi, 1985; Kawamura *et al.*, 1985).

Subsequently, a number of suppressor mutations for *rpoD47* were selected. The suppression of *rpoD47* could be readily detected on minimal glucose plates, since *rpoD47* strains produced small colonies and suppressed strains produced large colonies (Sun and Takahashi, 1985). Among the many suppressor mutations obtained, mutations in *spo0A*, *spo0D*, *spo0F*, and *spo0K* were found to be effective suppressors (Kawamura *et al.*, 1985). Thus the σ^{43} factor interacts either directly or indirectly with the products of a number of *spo0* genes. This in turn suggests that the major σ^{43} plays a major role in determining whether a cell will initiate a number of cellular responses to nutrient deficiency. For instance, the chemotaxis response including flagella formation, catabolite derepression (including internal metabolic reorganization and extracellular enzyme synthesis), asymmetrical membrane synthesis, and initiation of sporulation may depend on the interaction of the σ^{43} and the other nutrient sensing Spo0 proteins.

The Spo⁻ phenotype may be the result of mutations in transcriptional regulatory factors (i.e., *spo0* genes) which affect the transcription of genes involved in various membrane functions. Not all of the *spo0* functions are required for growth, but they may be necessary for sensing and responding to nutrient deprivation and for forming the special asymmetrical forespore membrane. The major role of σ^{43} and the Spo0A protein in nutrient sensing and catabolite repression is indicated by the Crs phenotype, which results from mutations in either of their genes.

7. REGULATORY EVENTS IN INITIATING SPORULATION

7.1. Replicative Activation of Sporulation

When cells were transferred from a rich medium to a sporulation resuspension medium which exerted nutritional stress on the cells (Sterlini and Mandelstam, 1969), sporulation was initiated only after a final round of DNA replication had been initiated (Mandelstam and Higgs, 1974). By tests at various times during synchronous DNA replication, it was determined that initiation of sporulation oc-

curred optimally when the DNA had replicated for about 15 min. This brought the replicating fork to the *cysA-sul^r* region of the chromosome. It was postulated that replication of the DNA molecule near a *spo0* gene would allow transcription to be initiated from this gene, which in turn triggered the whole sporulation response. The mechanism of transcriptional activation of sporulation during replication remains a hypothesis. No concrete evidence for this proposition has been found to date; however, *spo0H* and *spo0J* genes are in the regions of the *B. subtilis* chromosome when optimal time and bidirectionality of DNA replication are considered.

Since release from catabolite repression has also been postulated as a mechanism for initiating sporulation (Schaeffer *et al.*, 1965b), the relationship between catabolite-repressed enzymes and replication-activated sporulation was examined. It was found that although sporulation remained repressed unless chromosome replication was allowed to proceed, the derepression of histidase, sucrase, and α -glucosidase proceeded normally in the absence of DNA synthesis (Coote, 1974). It was thus possible to separate catabolite repression from replication-activated sporulation initiation, since catabolite derepression was not dependent on the replication phase of the chromosome (Coote, 1974). Furthermore, one of the earliest marker events in sporulation, the biosynthesis of the extracellular alkaline protease, is not related to the state of chromosome replication (Clarke and Mandelstam, 1980).

Catabolite repression, alkaline serine protease induction, and the repression of sporulation may be controlled by a common overall regulator, since they are initiated only after certain nutrients are depleted; however, the specific regulatory mechanisms for these phenomena are quite different as far as the requirement for DNA replication is concerned.

7.2. Role of Guanine Nucleotides

Although cells will not normally initiate sporulation or sporulate in the presence of rapidly metabolizable carbon, nitrogen, or phosphorus sources, the partial inhibition of the synthesis of GTP will cause massive sporulation even in the presence of favorable nutritional compounds (Freese *et al.*, 1979; Freese, 1981). Thus the effect of compounds which ordinarily cause catabolite repression of sporulation can be bypassed by conditions which reduce the GTP concentration of the cell. These conditions include the use of leaky purine-requiring auxotrophs (Freese *et al.*, 1978); inhibitors of purine biosynthesis, particularly of GTP (Mitani *et al.*, 1977; Heinze *et al.*, 1978; Lopez *et al.*, 1979); and stringent conditions of growth which increase ppGpp concentrations but which reduce GTP levels (Lopez *et al.*, 1981a,b). The stringent response is not in itself necessary for sporulation, since *relA* strains can sporulate in nutrient sporulation medium (Nishino *et al.*, 1979) or in synthetic medium containing decoynine, an inhibitor of GMP synthetase. Decoynine decreases the synthesis of guanine nucleotides but does not produce a stringent response (Lopez *et al.*, 1981b).

Catabolite repression does not prevent sporulation directly, since the expression of catabolite-repressed genes may be inhibited but sporulation can still be induced by the presence of decoynine (Lopez *et al.*, 1980).

All nutritional conditions which initiate sporulation have been correlated with a

decrease in GTP concentration of the cell, whereas other nucleotides have increased under some and decreased under other nutritional conditions (Freese, 1981).

A model has been proposed in which GTP either directly or indirectly serves as a corepressor to a repressor which binds to a methylated regulatory site on the DNA and prevents transcription of sporulation genes. When the GTP level is reduced, the repressor falls off allowing expression of the sporulation genes (Freese *et al.*, 1985).

Thus the initiation of sporulation could be a cascade of regulatory effects, each affecting several pathways of gene expression, but all leading ultimately to the control of expression of a key gene or set of genes.

7.3. Role of the Membrane

There is one common cellular location for a number of phenotypic aberrations in many early asporogenous strains, namely, the membrane. The major cytological effect of these mutations is the absence of asymmetrical forespore membrane formation, but there are a number of other effects such as lowered transformability (Schaeffer, 1969), absence of certain membrane proteins (Chui *et al.*, 1984), phenotypic reversion by polymyxin resistance (Guespin-Michel, 1971), bacteriophage tolerance (Ito, 1973), the absence of expression of extracellular enzyme genes (Hoch and Spizizen, 1969), the repression of chemotaxis and motility (Ordal *et al.*, 1985), and the possibility that *spo0A* controls expression of membrane protein genes (Ferrari *et al.*, 1985b).

A more precise link has been established between the development of competence for transformation and sporulation (Sadaie and Kada, 1983a). Competent cell formation was blocked by 0.7 M ethanol, which is a specific inhibitor of early events of sporulation, including forespore septum formation. The development of competence was also affected by several *spo0* mutations. Mutations in *spo0A*, *spo0B*, *spo0D*, *spo0E*, *spo0F*, *spo0H*, and *spo0K* repressed whereas mutations in *spo0C* (now known to be located in *spo0A*), *spo0G*, and *spo0J* had little effect on the development of competence. These results suggest that common steps are involved both in development of competence and in forespore septum formation and that competent cells are formed from early sporulating cells.

In addition, several septum-initiation (*div*) mutants, which exhibit filamentous growth at 45°C, were found to be defective in competence development and sporulation (Sadaie and Kada, 1983b). It has been proposed from these studies that some of the initial steps of septation are shared in competence development and in forespore septum formation. However, these two processes are distinct from the septation mechanism observed in vegetative cell division.

The fact that some chemotaxis (*che*) mutants are also asporogenous (Ordal *et al.*, 1985) suggests that alterations of membrane-associated functions could indirectly affect sporulation.

All the evidence suggests that either the initiation of sporulation is related directly to membrane functions or the regulatory mechanism that controls membrane functions also controls functions for the initiation of sporulation. Since the nutritional sensors of the cell, including those for chemotaxis, are associated with

the cell membrane, and the catabolite repression mechanism is suggested to be linked to these sensors, a picture is emerging as to how the environmental signals may be relayed from the membrane to the genome (see Section 8).

8. SUMMARY AND OVERVIEW OF THE CONTROL OF INITIATION OF SPORULATION

From various experimental approaches it was demonstrated that initiation of sporulation is affected by the nutritional environment of the cell (catabolite repression), the state of DNA replication, the level of GTP in the cell, the activity of *spo0* genes, the function of RNA polymerase holoenzymes, and the regulators of membrane genes and functions. Although all of these factors affect sporulation, some of the responses appear to be closer to the sporulation process than others.

The initiation of sporulation is controlled at various regulatory levels in the cell. Since sporulation is the final cellular response to nutrient deprivation, it is preceded by a number of cellular activities which attempt to alleviate the stressful situation prior to committing the cell to a state of dormancy.

The early responses include those mechanisms which attempt to overcome a deficiency in the nutrient supply for the cell. This includes the mobilization of the chemotaxis apparatus to "search" for more nutrients. This is accompanied by flagella synthesis, increased mobility, synthesis of a number of intracellular enzymes which can utilize the byproducts of growth and the synthesis of extracellular enzymes which can provide monomers from environmental biopolymers. If these responses do not improve the nutritional state of the cell, then sporulation is initiated. These responses could all be controlled by the mechanism of catabolite repression, which could be considered as part of the sensory device of the cell.

There is another link between chemotaxis and sporulation, since some *che* mutants are asporogenous. Is it possible that some *spo0* mutants are actually *che* mutants and that the whole sensing apparatus includes both types of functions? A systematic analysis of the *spo0* mutants for their chemotactic response may reveal this relationship. It is possible that some of the *che* and *spo0* genes code for transcription regulatory factors which control the expression of genes required for the sensing apparatus. If one considers catabolite repression as part of the sensory device, there appears to be a link between catabolite repression, Spo0 functions, Che functions, and RNA polymerase.

The initiation of sporulation and the other responses to nutrient deprivation are controlled by the direct or indirect action of *spo0* genes with the transcription machinery including the major RNA polymerase enzyme, $E\sigma^{43}$, and the minor σ enzymes. The *spo0* gene products are present in vegetative cells but are not necessary for growth. They appear to sense the availability of nutrients to the cell. When nutrients become depleted, these Spo0 products respond by interacting (directly or indirectly) with the RNA polymerase holoenzymes to trigger the expression of genes involved in metabolism (catabolite derepression of intracellular enzymes) and membrane functions such as motility (flagella formation), extracellular enzymes (hydrolytic enzymes), and sporulation genes (asymmetrical forespore membrane formation).

GTP (or other guanine nucleotide derivatives) interacts with either Spo0 products, RNA polymerase, or other regulatory proteins and acts as a coeffector in gene repression or derepression. This level of activity is more directly associated with the expression of sporulation genes than with catabolite-repressed genes, since the reduction of intracellular levels of GTP can result in sporulation in the absence of catabolite derepression of genes.

A mutation in a *spo0* gene reduces the cell's ability to respond to environmental changes and has a pleiotropic effect, since the cascade of membrane-controlled functions is not initiated. However, some of the *spo0* mutations can be compensated by a mutation (*rpoD47*) in either the σ^{43} gene or in *spo0A*, indicating that interactions are occurring between the products of these two genes and the other *spo0* genes. The mutations in *spo0* genes are also suppressed by a mutation in *spo0A* (*sof*). Thus σ^{43} and Spo0A protein carry out major regulatory roles for the initiation of nutrient stress responses and both functions are essential for this purpose. Mutations in either gene can result in the Crs phenotype, indicating a functional link between σ^{43} and the Spo0A protein. It is interesting that mutations in *spo0A* cannot be suppressed by the *rpoD47* (*crsA*) mutation, although the reverse is true.

A number of phenomena associated with the initiation of sporulation have been shown to be in separate functional pathways (see Fig. 3). Catabolite derepression of a number of genes is the major manifestation of carbohydrate and nitrogen depletion of the medium. The control of catabolite repression and the expression of the subtilisin gene have been dissociated from the specific pathway of sporulation; sporulation can be initiated while catabolite-repressed genes are still repressed

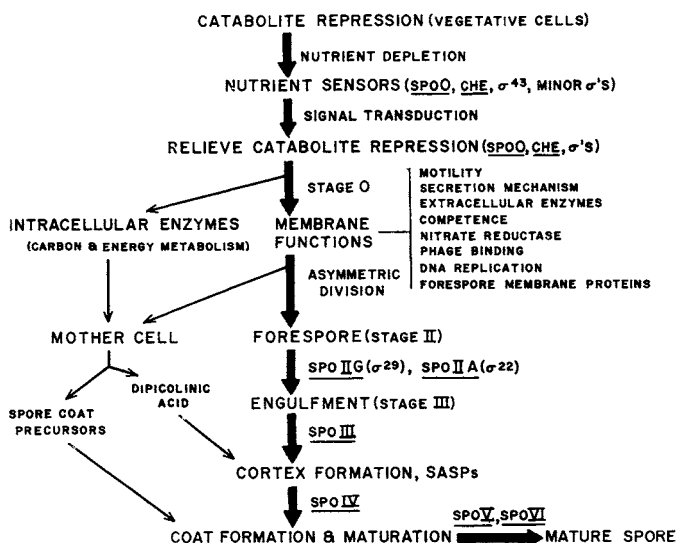


Figure 3. A summary of some of the events and proposed regulatory mechanisms during initiation and the various stages of sporulation in *B. subtilis*. See text for the discussion of the proposed control elements of sporulating *Bacillus*.

and when the subtilisin gene has been eliminated from the chromosome by deletion. Furthermore, while the initiation of sporulation requires a particular stage of chromosome replication, catabolite-repressed genes can be expressed at any stage of chromosome replication. Although aberrations in the overall regulatory function can affect these multiple pathways simultaneously, the specific functions required for the sporulation process can be delineated from those of others.

A much better understanding of the roles and relationships among catabolite repression, chemotactic responses, membrane functions, *spo0* genes and their products, and RNA polymerase and its transcription factors in the initiation process of sporulation will be obtained in the near future. In addition, the identification of many more sporulation and germination genes and their protein products appears imminent.

One of the possible practical uses that might be derived from the studies on the transcription system of sporulating cells is the utilization of promoters for the expression of foreign genes in *B. subtilis* during the stationary phase. If these promoters are also fused to signal peptide sequences and foreign genes, it may be possible to synthesize and secrete foreign proteins into the medium during sporulation (Doi and Wang 1986).

Thus the application of recombinant DNA technology has provided much novel information concerning sporulation and has opened a new vista on the roles of various interrelated factors during the initiation of sporulation. A more precise definition of the sporulation genes and their mechanisms of action should soon be forthcoming.

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The *Bacillus* Cell Envelope

9

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1. INTRODUCTION

The primary function of the bacterial cell envelope is to protect the underlying protoplast and its fragile membrane from mechanical assault and from swelling and disruption due to the unrestricted inflow of water. The high surface-to-volume ratio that results from the very small size of bacteria is important in permitting the high transfer rate of nutrients needed to maintain the rapid metabolic activity that enables some bacteria to double in under 20 min in appropriate environments. The high internal concentration of metabolites necessary to sustain this rapid growth results in differences in osmolarity between the protoplast and the relatively dilute media in which bacteria commonly grow. A consequent influx of water is prevented by the envelope, which resists the necessary increase in protoplast volume. In cocci, the envelope shape is that which most efficiently withstands the hydrostatic pressure. In rod-shaped bacteria, the envelope has additionally to maintain the less stable rod shape, resisting the rounding effects of the internal hydrostatic pressure. The work needed to resist the rounding forces is presumably offset by advantages arising from the increased surface area of the rod-shaped cells (Koch, 1983).

The component primarily responsible for the shape and mechanical strength of the envelope is peptidoglycan, a heteropolymer found only in prokaryotes (Rogers *et al.*, 1980). In gram-negative bacteria the peptidoglycan is present as a fairly thin layer overlying the protoplast membrane but is itself covered by an outer membrane that forms an important selective permeability barrier that not only excludes potentially damaging antibiotics and hydrolytic enzymes but also traps a number of protective and metabolically important enzymes within the periplasmic gel framework of the peptidoglycan (Hobot *et al.*, 1984). Gram-positive bacteria do not have the outer membrane structure but the peptidoglycan layer, which contains a number of covalently associated anionic polymers, is a very much thicker structure containing several sheets of peptidoglycan. Because of the lack of an outer membrane permeability barrier, the envelope of gram-positive bacteria does not provide the protoplast with quite as effective protection from inhibitory agents as does that of gram-negative bacteria, though it is now realized that the protection

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given to the protoplast is not solely mechanical and that the ion exchange and sieving properties of the wall can modulate the effect of environmental agents. Thus large molecules are excluded (Scherrer and Gerhardt, 1971), and there is evidence (Chase and Reynolds, 1981) that some protective enzymes may remain associated in the peptidoglycan gel so that their effect is concentrated at the cell surface, a process that will be more efficient than excretion into bulk culture fluid. Nevertheless, the gram-positive envelope appears as a structurally less complex entity, and the peptidoglycan component that surrounds the protoplast membrane can be quite easily isolated, together with the covalently attached associated polymers. This structure is generally referred to as the cell wall. It retains the shape of the cell and is composed of peptidoglycan (40% or more by weight) to which other polymers such as teichoic or teichuronic acid are covalently attached (Shockman and Barrett, 1983).

The wall in most strains of the genus *Bacillus* is a thick elastic structure that surrounds the protoplast and protects it from internal osmotic pressures that can reach 20–25 atmospheres (Mitchell and Moyle, 1956). The wall is responsible for the surface properties and for the shape of the bacteria, and it is important in relation to methods for immobilizing, harvesting, and disrupting bacteria that are being used in commercial processes. The way in which bacilli are grown affects the composition and structure of the wall (Ellwood and Tempest, 1972) and this can result in changes in surface charge, in cell separation or filamentation, and in specific surface properties such as the ability to bind phages. The wall has ion exchange properties and porosity characteristics that may affect the secretion of proteins or other exported products, and the effect of growth conditions on these and on its susceptibility to autolysis can also be important.

In stained thin section the wall appears to be fairly amorphous, although three-banded structures with densely staining inner and outer layers are frequently seen. This contrast pattern probably reflects differences in packing of wall material rather than concentration of any particular component polymer at inner and outer layers (Millward and Reavely, 1974), although some strains do have a regularly arranged protein layer covering the outer surface (Sleytr, 1983).

The protoplast membrane is shown by electron microscopy as a typical bilayer structure, 7–8 nm thick, surrounded by the 20- to 50-nm-thick cell wall. These structures are closely apposed so that resolution between the outer surface of the membrane and the inner surface of the wall can be difficult. This reflects an intimate physical and chemical association between the membrane and the wall that has an important bearing on envelope function. The central role of the membrane in the metabolic activities of the cell is well documented and outside the scope of this chapter, but three aspects of membrane function are particularly relevant to the topics considered here, i.e., the role of the membrane in wall synthesis and assembly, in protein secretion, and in the coordination of genome segregation with cell division. These topics are discussed below. It should be noted that in many cases the functional entity is the whole wall-membrane system, i.e., the envelope, and that changes in composition of the membrane can have major effects on the properties of other components of the envelope. One example is given by the effect of cerulenin, which inhibits fatty acid synthesis on *B. subtilis*. Appropriate concentrations permit growth of bacilli but reduce the phospholipid content to half. Apparently as a consequence, the bacteria grow in chains of deformed cocci that are

resistant to β -lactam-induced lysis (Rogers and Thurman, 1985). Overproduction of a membrane component has also been suggested as the reason for both the Lyt- phenotype and the massive overproduction of levansucrase in *sacU^h* mutants of *B. subtilis* (Chambert and Petit-Glatron, 1984).

2. THE PROTOPLAST MEMBRANE

2.1. Lipid Components

The cytoplasmic membrane in *Bacillus* is rich in phospholipids and proteins but, like that in most other bacteria, it lacks sterols and polyunsaturated fatty acids and the range of phospholipids is limited (Bishop *et al.*, 1967). Phosphatidylethanolamine occurs in all members of the genus and constitutes between 20 and 40% of the total phospholipid, other components being cardiolipin and phosphatidylglycerol. O-Aminoacyl derivatives of phosphatidylglycerol are found in bacteria growing under acidic or slightly acidic conditions (Goldfine, 1972); these include alanyl (in *B. cereus*), lysyl (in *B. megaterium* and *B. subtilis*), and ornithyl (in *B. natto*) phosphatidylglycerol. Glycosylated derivatives of phosphatidylglycerol are also found as minor components in *B. megaterium* and *B. subtilis*. The most common form of extractable glycolipid is the glycosyl diglyceride (Shaw, 1970), which occurs both free and as part of the lipoteichoic acid molecule (Button and Hemmings, 1976b) for which it provides an anchor in the membrane (Lambert *et al.*, 1977). Although lipoteichoic acids are primarily components of the membrane, they are excreted into the culture fluid and can be important wall and surface components in some bacteria. They are considered in more detail below.

Most species produce little, if any, unsaturated fatty acid. Branched fatty acids (anteiso- and both odd- and even-numbered iso-) normally constitute 60–90% of the total (Kaneda, 1977) together with smaller proportions of members of the straight-chain series. The proportions of these four series can vary depending on genetic and environmental factors. The branched fatty acids appear to fulfill the role taken by unsaturated fatty acids in other organisms and are involved in maintaining membrane fluidity, organization, stability, and function (Boudreaux and Freese, 1981). Thus membranes of *B. megaterium* grown at low temperature contain increased amounts of branched and short-chain fatty acids but lower proportions of acidic phospholipids. Growth at low temperature also results in changes in protein content and these changes give membranes with different mechanical properties and permeabilities (Eisenberg and Corner, 1978). Isoprenoid lipids, present in small proportions in the membrane, have the important function of acting as the lipid "carrier" in the biosynthesis of wall polymers.

2.2. Protein Components

The high protein content (50–70%) of bacterial membranes is thought to reflect the necessity for all membrane functions to be carried out in the relatively undifferentiated cytoplasmic membrane although, despite an absence of clear-cut

morphological distinction, some differentiation in composition and function is apparent from recent work.

Membrane from *B. subtilis* (Marty-Mazars *et al.*, 1983) can be separated into a fraction complexed with ribosomes and a fraction free of ribosomes. By analogy with the membranes of the smooth and rough endoplasmic reticulum in animal cells, it might be expected that these complexed and free membrane fractions would differ in only a very few protein bands. However sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) reveals six major bands (M_r 31–130 kDa) that are unique to the complexed membrane fraction. One (but only one) of these has been linked to protein secretion since it is covered by attached ribosomes. The membrane fractions may differ in other functions besides protein secretion since the free membrane fraction also contains unique proteins, including the penicillin-binding proteins (*B. subtilis* contains five, two of which can be further resolved) that catalyze crosslinking and reshaping reactions in wall assembly.

Fragmentation of the membrane thus gives separate vesicles for the domains involved in protein translocation and those involved in synthesis and assembly of peptidoglycan. These domains of different function may be distributed as a mosaic or they may represent different zones in the membrane. Differences in composition have been noted between whole-cell and minicell membranes indicating that there might be some differentiation between polar and cylindrical regions in the membrane (Shohayeb and Chopra, 1985). Since wall synthesis proceeds more slowly at the cell pole than in cylindrical regions of growing bacilli, and membranes from minicells have diminished proportions of penicillin-binding proteins, it may be expected that membrane at the cell poles will contain reduced proportions of wall synthetic sites.

2.3. Lipoteichoic Acid

Like most other gram-positive bacteria, strains of *Bacillus* contain substantial proportions (about 1–2% by weight) of lipoteichoic acid. This is held mostly in association with the cytoplasmic membrane but may also be present in either acylated or deacylated forms in the wall, at the surface, and excreted into the culture fluid (Wicken and Knox, 1975, 1980).

The lipoteichoic acids found in *Bacillus* are similar in structure to the 1–3 polyglycerol phosphate teichoic acids that occur in the walls of many species, but with two major differences. The first is that lipoteichoic acid is attached through a phosphodiester linkage to a glycolipid or acylglycerol that anchors it in the membrane (Button and Hemmings, 1976a; Iwasaki *et al.*, 1986) rather than to a linkage unit of the kind that holds wall teichoic acid to peptidoglycan (see Section 3.2.1). The second major difference (see Koch and Fischer, 1978) is that the stereochemistry of the glycerol phosphate is opposite in the two polymer types. Though most immediately apparent at the glycerol terminal end of the chain, i.e. the end distal to the glycolipid or linkage unit which terminates in either an *sn*-glycerol-3-phosphoryl unit (wall teichoic acid) or an *sn*-glycerol-1-phosphoryl unit (lipoteichoic acid) as in Fig. 1, the whole polymer chain is asymmetrical and the two types of teichoic acid will be recognizably different over regions that include either end of the chain. The difference in stereochemistry between wall and membrane glycerol teichoic acids arises from their different biosynthetic routes, i.e., wall teichoic acid

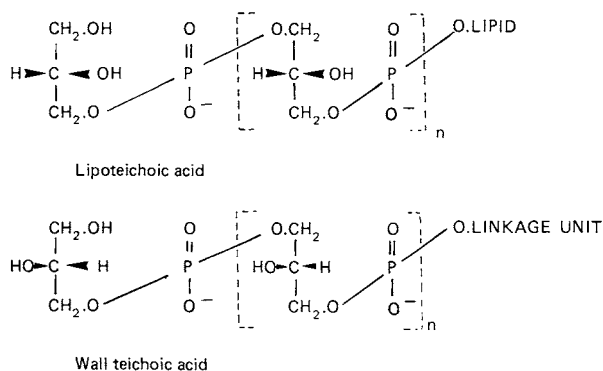


Figure 1. Stereochemistry of the glycerol phosphate residues in membrane lipoteichoic acid and in wall teichoic acid.

from cytidine diphosphate (CDP) glycerol (Burger and Glaser, 1964) and lipoteichoic acid from phosphatidyl glycerol (Edmur and Chui, 1975). The functional significance of this difference in stereochemistry is not yet clear.

Like wall teichoic acids, lipoteichoic acids usually carry glycosyl or D-alanyl substituents at the 2 positions of a proportion of the glycerol units. The lipoteichoic acids in *B. subtilis*, *B. licheniformis*, and *B. pumilis* contain D-alanyl, D-glucosyl, and D-glucosaminyl substituents and are anchored in the membrane by attachment to gentiobiosyldiacylglycerol. In *B. coagulans* and *B. megaterium* the lipoteichoic acids contain D-galactosyl substituents, no alanine, and are joined directly to diacylglycerol with no intervening sugar (Iwasaki *et al.*, 1986).

The D-alanyl substituents in teichoic acid modulate its ability to bind cations and may be involved in regulating the magnesium-buffering capability at the membrane (Hughes *et al.*, 1973); they also modulate the inhibitory effect of lipoteichoic acid on autolysins (Fischer *et al.*, 1981), though it is not known whether this has any physiological significance in *Bacillus*.

Work with other bacteria has shown that biosynthesis of lipoteichoic acid proceeds by transfer of *sn*-glycerol-1-phosphate units from phosphatidylglycerol to a glycerol glycolipid and subsequently to the glycerol terminal end of the nascent chain (Ganfield and Pieringer, 1980; Taron *et al.*, 1983). The products of the reaction are the growing lipoteichoic acid and a 1,2-diglyceride that may then be phosphorylated to phosphatidic acid, a precursor of phosphatidylglycerol, so recycling the diglyceride moiety of the phospholipid. Phosphatidylglycerol is present in two distinct pools in *B. megaterium*, one of which exhibits metabolic stability whereas the other undergoes rapid turnover and may be the source of the glycerol units for lipoteichoic acid synthesis (Lombardi and Fulco, 1980; Lombardi *et al.*, 1980). Taron *et al.* (1983) found that inorganic phosphate stimulates both the synthesis of phosphatidylglycerol and the rate of chain extension of lipoteichoic acid. This may partly explain the observation that phosphate-limited *B. licheniformis* contains only one-tenth as much lipoteichoic acid as is present in the bacteria grown under other conditions.

The mechanism of incorporation of alanine ester has not been studied in *Bacillus*, but work with other bacteria (Childs and Neuhaus, 1980) shows that incorporation of alanine ester proceeds by a two-step reaction in which D-alanine is first

activated by reaction with ATP, forming D-alanyl-AMP, and then transferred to a lipoteichoic acid acceptor in the membrane. The D-alanyl esters can be transferred within lipoteichoic acid and from lipoteichoic acid to wall teichoic acid by inter- and intrachain transacylation reactions, so that perturbation of the D-alanine ester content at one location in the envelope can be translated to another location by transacylation of the alanine. Neuhaus (1985) suggested that this could be a way of transmitting a signal from one location to another.

3. WALL STRUCTURE AND ORGANIZATION

3.1. Peptidoglycan

3.1.1. Composition and Structure

Approximately half of the weight of the cell wall in gram-positive bacteria is peptidoglycan. Peptidoglycan consists of linear chains of glycan held together by crosslinked peptides. The glycan is composed of regularly alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid which are held together by β -(1-4) glycosidic linkages. The carboxyl group of each muramic acid residue forms an amide linkage with the N-terminal amino acid of a short peptide which may be crosslinked to a corresponding peptide on a neighboring glycan so linking the glycan chains together. Since each glycan chain carries several peptide substituents, it can become linked to more than one neighboring glycan chain so that an enormous macromolecule forms. This "bag-shaped macromolecule" (Weidel and Pelzer, 1964) may comprise essentially all of the peptidoglycan in the wall, giving a structure that combines great strength with considerable elasticity so that the wall can shrink or swell in response to changes in pH, ionic strength, or osmolarity.

In the vast majority of strains of *Bacillus* (Schleifer and Kandler, 1972) the peptide consists of chains in which L-alanine, connected to the carboxyl group of the muramic acid through an amide linkage, is linked through its carboxyl group to the amino acid group of D-glutamic acid. The γ (not the α) carboxyl group of glutamic acid is linked to the amino group at the L-center of diaminopimelic acid and the carboxyl group at this center is linked to D-alanyl-D-alanine.

Crosslinking is accomplished by transpeptidation reactions in which the terminal D-alanine is removed from the pentapeptide and the D-alanine at position 4 of this "donor" peptide becomes linked to the amino group of the diaminopimelic acid residue in the "acceptor" peptide (Fig. 2). Terminal alanine residues may also be removed by D-alanine carboxypeptidases: peptides from which terminal alanine residues are removed can no longer act as donor peptides and the carboxypeptidases may act to tailor crosslinkages within the glycan (Ghuysen, 1977). The distribution of tri-, tetra-, and pentapeptides differs in different strains, as does the extent of crosslinking, which ranges from a fairly low value of 30% (i.e., rather more than two-thirds of the diaminopimelic acid residues have a free amino group and are not involved in crosslinking) up to 60% in *B. cereus*.

Other differences in structure include the amidation of carboxyl groups in the peptides. In most cases only one of these is modified in this way. In *B. licheniformis*

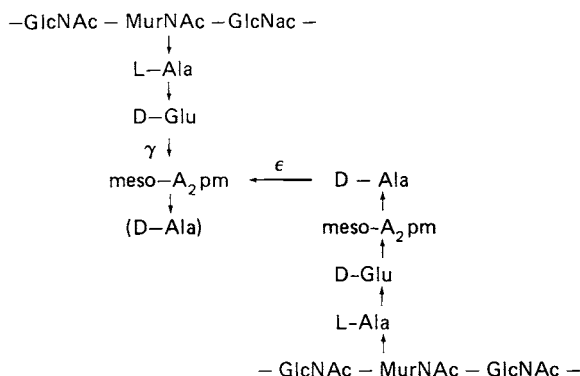


Figure 2. Crosslinkage of “donor” and “acceptor” peptides in *B. subtilis* peptidoglycan.

the α -carboxyl group of the D-glutamic acid is amidated whereas in *B. subtilis* it is the second carboxyl group of the *meso*-diaminopimelic acid that is amidated. In effect, such amidation neutralizes the acidic carboxyl group and will reduce the charge density in the peptidoglycan; however, the structural or physiological consequences of this are not yet understood. Some *Bacillus* strains, e.g., *B. firmis*, *B. lentus*, and *B. megaterium*, have no such amide groups.

In some strains a small proportion of the peptidoglycan chains may be crosslinked differently. Thus the presence of a small amounts of the D,D isomer of diaminopimelic acid in *B. megaterium* and *B. cereus* may be explained by crosslinking units in which two primary peptide chains are linked together through their D-alanine residues, at position 4 to a “bridging” molecule of D,D-diaminopimelic acid (Van Heijenoort *et al.*, 1969; Wickus and Strominger, 1972). A more marked structural difference has been found in the peptidoglycan of a few bacilli that are characterized by the production of spherical endospores (see Section 4.3.1, Chapter 2). Thus *B. pasteurii* and *B. sphaericus* contain lysine rather than diaminopimelic acid in the wall peptidoglycan, even though the latter amino acid is present in the peptidoglycan of the spore cortex (Powell and Strange, 1957). In these peptidoglycans, adjacent peptides are linked through bridge units of D-aspartic acid or L-alanyl-D-aspartic acid extending between the 4 (D-alanine) and the 3 (L-lysine) positions of the primary peptides. Although *meso*-diaminopimelic acid is made by all bacteria as the biosynthetic precursor of lysine, bacilli that have lysine in their peptidoglycan make diaminopimelic acid by a different route from that used by the more commonly encountered bacilli that have diaminopimelic acid in their peptidoglycan (Bartlett and White, 1985).

3.1.2. Organization in the Wall

The overall strength of peptidoglycan is a function of the extent of crosslinking and the glycan chain length. The averaged values of glycan chain length and of peptide crosslinking that can be obtained by present analytical methods give little information on how uniform these are within the wall. So far neither the different shape nor the different susceptibility to autolytic enzymes of polar and cylindrical

regions of the cell wall can be related to observable differences in chemical structure. Nevertheless some information on the arrangement of peptidoglycan in the wall is now available.

Other than strength, perhaps the most important feature of peptidoglycan is its elasticity. This was well established in an early, important series of investigations showing that walls could shrink and swell in response to their ionic environment (Marquis, 1968; Ou and Marquis, 1970), and by work showing changes in the organization of components within the wall and at the surface that could be explained on the basis of electromechanical interactions within a polyelectrolyte gel structure for the peptidoglycan (James and Brewer, 1968).

As would be expected for a molecule having such physical properties, the highly ordered semicrystalline arrangement responsible for the strength and rigidity of chitin or cellulose is not found in peptidoglycan. The latter gives X-ray diffraction patterns that show very much lower levels of order, though the structure does not seem to be entirely amorphous (Burge *et al.*, 1977a,b; Labishinski *et al.*, 1979). There is good evidence for a layered structure in the wall made of several sheets of peptidoglycan. Within these sheets the glycan strands are arranged more or less parallel to each other, possibly perpendicular to the long axis in a circular arrangement like the hoops round a barrel or possibly in a spiral or corkscrew winding in which strands are at an angle to the perpendicular (see Verwer *et al.*, 1978). A helical arrangement of peptidoglycan strands along the cylindrical axis of the cell is compatible with the helical morphology shown by filaments of *Lyt*⁻ mutants of *B. subtilis* (Mendelson, 1976; Tilby, 1977). Based on an average chain length of 100 disaccharide units for the glycan, the length of each chain would be approximately 0.1 μm so that about 16 chains, end to end, would be needed to circumscribe the wall (Ward, 1973).

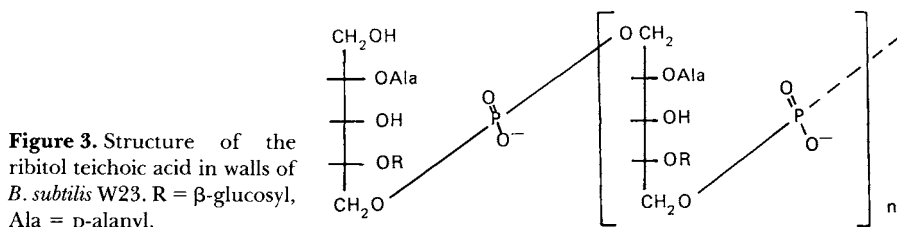
Modeling studies using conformation energy calculations show that favored conformations of the glycan strands will be those in which adjacent sugars can show different degrees of twist but arranged so that overall the strands have an almost fully extended conformation, forming rigid rod-shaped macromolecules from which the peptide side chains can emerge at different angles (Barnickel *et al.*, 1983). Crosslinkages can form between peptides that extend between neighboring glycan strands within the same plane so that adjacent glycan strands become interconnected through flexible peptides. Infrared spectroscopy studies exclude a highly ordered structure for the peptides as well as for the glycan and indicate the absence of regular conformations such as α helices or β -pleated sheets (Nauman *et al.*, 1982). Minimum energy calculations (Barnickel *et al.*, 1983) show that these peptides will adopt a ringlike conformation due to the γ -bonded glutamic acid and the alternating L- and D-amino acid residues. However, the most stable ringlike conformation can be unwound by mechanical forces to give more extended arrangements. The extended glycan strands thus provide elements of rigidity within the elastic peptidoglycan network but they are connected by flexible peptides that can be stretched out so that the distance between adjacent glycan strands can change markedly. Because of the twisted conformation of the glycan, peptides can extend outwards above and below the plane of the peptidoglycan layer and crosslinkage between such peptides could serve to connect the different layers of peptidoglycan within the multilayered structure of the wall.

3.2. Anionic Polymers

Walls of most strains of *Bacillus* contain teichoic acid or teichuronic acid, held in place by covalent attachment to peptidoglycan (Ward, 1981). These anionic polymers can make up about half the material in the wall. Although they are often described as "accessory" or "secondary" wall polymers, their presence is required for the ordered assembly of wall material and for normal wall function. Electrostatic interactions involving charged groups in these polymers contribute to the overall polyelectrolyte gel structure of the wall and mutants blocked in the synthesis of adequate amounts of anionic polymers grow with disturbed morphology as irregular coccoid forms (Forsberg *et al.*, 1973). Teichuronic acids contain charged carboxyl groups whereas teichoic acids contain charged phosphate groups and, often, charged amino groups present in *D*-alanine ester substituents. Anionic polymers contribute to surface charge and also to specific surface properties. They interact with autolysins and other proteins, and can hold these in the wall and modulate their activity. The relative proportions of the individual polymers present vary greatly according to growth conditions, which can therefore give rise to substantial changes in wall and surface chemistry.

3.2.1. Teichoic Acids

Glycerol teichoic acids are widely distributed in walls of *Bacillus* species but ribitol teichoic acids has been reported only in some strains of *B. subtilis*. The polyribitol phosphate teichoic acid in the wall of *B. subtilis* W23 was the first teichoic acid to be studied in detail (Armstrong *et al.*, 1960). It consists of chains of about 35 ribitol phosphate units linked through phosphodiester bonds between the 1 and 5 positions of adjacent units. In most of the chains each ribitol unit has a β -*D*-glucopyranosyl substituent at the 2(L) position (Fig. 3) but some chains lack these substituents. Individual chains are homogeneously substituted—either fully or not at all—depending on whether glucosyltransferase is a component of the biosynthetic enzyme system. *D*-Alanine ester substituents are also attached to some of the ribitol units, probably at the 2(D) position. The amino group of the alanine is protonated under physiological conditions and so can neutralize an adjacent phosphate. The ester linkage is remarkably labile to alkali, like that in aminoacyl-RNA, because of the adjacent phosphate and hydroxyl groups (Shabarova *et al.*, 1962). A consequence of this lability is that the alanine esters are easily lost during wall isolation procedures and during growth at alkaline pH values.



A variety of different structural types of glycerol teichoic acid has been reported. *Bacillus subtilis* strains 168 and 3610 and *B. cereus* AHU 1030 contain 1,3-linked polyglycerol phosphate with α -linked glucose substituents (Birdsell *et al.*, 1975; Sasaki *et al.*, 1983). *Bacillus subtilis* var. *niger* contains a 1,2-linked polyglycerol phosphate teichoic acid with β -D-glucopyranosyl substituents (de Boer *et al.*, 1978). Walls of *B. licheniformis* ATCC 9945 contain polyglucosylglycerol phosphate teichoic acids in which the sugar substituents form an integral part of the polymer chain: units of β -galactosylglycerol phosphate and α -glucosylglycerol phosphate connected through phosphodiester linkages between the glycerol and sugar moieties of adjacent units are present in separate chains (Burger and Glaser, 1966). Sugar substituents also form an integral part of the polymer chain in the wall teichoic acids of *B. stearothermophilus* B65 (de Boer *et al.*, 1978), *B. subtilis* AHU 1035 and 1037 (Kaya *et al.*, 1984), *B. licheniformis* AHU 1371 (Kaya *et al.*, 1984), and *B. coagulans* AHU 1366 and AHU 1634 (Kaya *et al.*, 1985, Kojima *et al.*, 1985c). Walls of *B. pumilis* AHU 1366 contain a poly-*N*-acetylglucosamine-1-phosphate teichoic acid that does not contain glycerol in the repeating unit (Kojima *et al.*, 1985b), although small amounts of glycerol teichoic acid are also present in the wall.

Mixtures of teichoic acid are often encountered. Small amounts of 1,3-polyglycerol phosphate teichoic acid are commonly present in walls that contain 1,2-linked teichoic acid or teichoic acids where sugars form an integral part of the chain. Walls of *B. subtilis* 168 contain 1,3-polyglycerol phosphate, polyglucosyl-*N*-acetylgalactosaminyl-1-phosphate, and an additional polymer containing galactosamine (Duckworth *et al.*, 1972). Several species also contain teichuronic acid in addition to teichoic acid. It is not known whether the presence of a mixture of anionic polymers is functionally significant.

Teichoic acid is held in the wall by covalent attachment to peptidoglycan through a "linkage unit" (Coley *et al.*, 1978) composed of a short chain of glycerol phosphate attached to a disaccharide (Kojima *et al.*, 1985a) that is linked through a sugar-1-phosphate to muramic acid (Fig. 4). Sugar-1-phosphate linkages are labile in dilute acid and the linkage between glycerol phosphate and disaccharide is labile in dilute alkali so that teichoic acid is readily extracted by either acid or alkali (see Coley *et al.*, 1978).

Ito and his colleagues carried out detailed studies on the structure of linkage units in several species and found glycerol phosphate oligomers containing up to seven units (Kojima *et al.*, 1985a). The disaccharide *N*-acetylmannosaminyl-*N*-acetylglucosamine occurs widely as an essential component of the linkage units for teichoic acids (Sasaki *et al.*, 1983, Kojima *et al.*, 1985a) but an alternative disac-

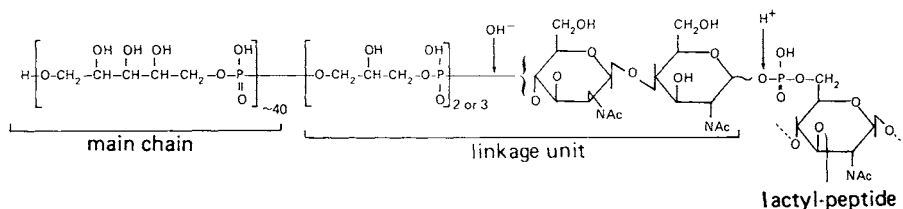


Figure 4. Linkage between teichoic acid and peptidoglycan in *B. subtilis* W23.

charide, glucosyl-*N*-acetylglucosamine, occurs in a group of *B. coagulans* (Kaya *et al.*, 1985).

3.2.2. Teichuronic Acids

In addition to teichoic acids, walls of *B. licheniformis* grown in batch culture contain teichuronic acid composed of glucuronic acid and *N*-acetylgalactosamine (Janczura *et al.*, 1961). Similar teichuronic acids are present in walls of various strains of *B. subtilis* grown under phosphate limitation. Those in *B. subtilis* W23 (Wright and Heckels, 1975) and *B. licheniformis* 6346 (Hughes and Thurman, 1970) both consist of linear chains of a repeating disaccharide unit (Fig. 5). The same component sugars are present in the teichuronic acid of *B. licheniformis* 9945 but detailed chemical and physical studies (Lifley *et al.*, 1980) revealed a more complex repeating unit. The teichuronic acid in walls of *B. subtilis* AHU 1031 is also complex in structure and is particularly interesting in that it contains both carboxyl and phosphate groups in the same molecule. The uronic acid is *N*-acetylmannosaminuronic acid, also found in some strains of *B. megaterium* and *B. polymyxa* (Yoneyama *et al.*, 1982). The repeating unit (Fig. 6) contains glucose, *N*-acetylmannosaminuronic acid, and *N*-acetylglucosamine with glucose branches, about half of which carry glycerol phosphate units, joined to the *N*-acetylglucosamine units (Yoneyama *et al.*, 1984). The polymer consists of some 12–15 repeating units, terminating at the reducing end in an *N*-acetylglucosaminyl residue that is probably linked through a sugar-1-phosphate linkage to muramic acid phosphate. The walls also contain substantial proportions of a glucogalactan with a molecular mass of 100 kDa.

Teichuronic acids (and teichoic acids) also occur in walls of alkaliphilic bacilli (Aono and Horikoshi, 1983; Aono, 1986). Walls of the bacteria grown under alkaline conditions contained more teichuronic acid (and also more of a second polymer composed of glucuronic acid and glutamic acid) than did those of bacteria grown at neutrality (Aono, 1985).

Teichuronic acid is held in the wall by covalent attachment to peptidoglycan. In *B. licheniformis* the linkage does not involve a specialized unit of the type found with teichoic acids but instead consists of a sugar-1-phosphate linkage between the reducing terminal *N*-acetylgalactosamine and the primary hydroxyl group of an *N*-acetylmuramic acid residue in the peptidoglycan (Wyke and Ward, 1977b; Ward *et al.*, 1980). The teichuronic acid in walls of *B. subtilis* AHU 1031 consists of some 12–15 repeating units terminating in an *N*-acetylglucosamine residue that is probably linked through a sugar-1-phosphate linkage to muramic acid (Yoneyama *et al.*, 1984).

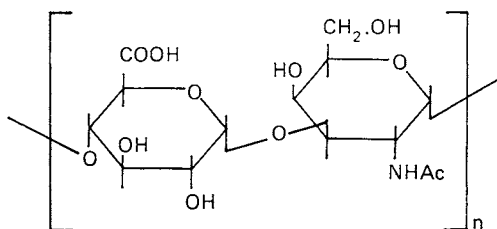


Figure 5. Repeating structure in the teichuronic acid of *B. subtilis* W23.

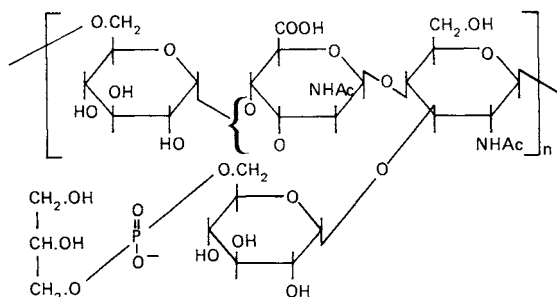


Figure 6. Repeating structure in the complex teichuronic acid of *B. subtilis* AHU 1031.

3.2.3. Location of Anionic Polymers in the Wall

Most models of wall structure in gram-positive bacteria represent the teichoic acid chains extending radially through the wall to, and outward from, the outer surface. It is not known whether the *N*-acetylmuramic acid residues to which teichoic acid chains are attached occupy a special location or whether they are distributed randomly along the glycan chain. However, it is known that individual glycan chains carry only one type of anionic polymer.

Studies by Ward and his colleagues in *B. licheniformis* 6346 (see Ward and Curtis, 1982) showed that teichoic and teichuronic acids are present on separate glycan chains: walls in which these chains have not been fragmented by autolysis contain only a small proportion of glycan (less than 15%) that is not linked to anionic polymer. Essentially, therefore, the peptidoglycan is present as a peptidoglycan-anionic acid polymer complex. It follows that teichoic and teichuronic acids cannot be restricted to particular locations, such as the outer "layer" of the wall, but must instead be joined to peptidoglycan chains throughout the thickness of the wall. This is obviously so, too, from the wall assembly process in bacilli which involves the incorporation of new wall material (i.e., peptidoglycan-anionic polymer complex) at the inner surface of the wall cylinder from where it moves to the outer surface as a consequence of growth (Section 4.4).

A great deal of evidence shows that much of the teichoic acid in the wall is exposed at the cell surface, where it can participate in binding antibody molecules, lectins, and phages. Electron microscopic examination of sections of *B. subtilis* 168 that had been allowed to bind concanavalin A revealed a discontinuous "fluffy" layer of adsorbed lectin extending some 40 nm out from the "surface" of the wall. This is thought to represent extended chains of α -glucosylated teichoic acid extending outward from the surface of the wall (Birdsell *et al.*, 1975; Doyle *et al.*, 1975) and held together by the bound concanavalin A molecules.

Attempts to estimate what proportion of the total teichoic acid is surface-exposed have been reported by various authors who measured the amounts of antibody or lectin bound by walls, enzymically solubilized walls and walls that have been incubated briefly with hydrolases to loosen the peptidoglycan framework (Burger, 1966; Doyle *et al.*, 1975). Interpretation of such studies is complicated because it is likely that binding of the probes used is limited by steric crowding at the surface rather than by the amount of teichoic acid present (Lang and Archibald,

1983). Nevertheless, these studies all indicate that some of the teichoic acid present is inaccessible to probes at the surface. This is shown most clearly by experiments in which *B. subtilis* was grown under phosphate limitation, so that the walls contained teichuronic acid and no teichoic acid, and then changed to phosphate-rich medium so that the newly synthesized material contained teichoic acid. This new material is located in the inner region of the wall and could not be detected at the bacterial surface by either phages (Archibald and Coapes, 1976) or concanavalin A (Anderson *et al.*, 1978b) until it had moved to the outer surface as a consequence of growth. The results show that about half of the anionic polymer in the wall is located below the surface and inaccessible to macromolecular probes.

Much less teichoic acid is detected at the inner than at the outer surface of isolated walls, indicating differences in structure between these wall regions. Thus isolated walls of *B. subtilis* bind little more specific antibody than do whole cells (Burger, 1966), they show a "fuzzy" layer of bound concanavalin A at the outer surface but not at the inner surface (Birdsell *et al.*, 1975), and they bind cationized ferritin at the outer but not the inner surface, though they do bind the ferritin at both surfaces after brief autolytic digestion (Sonnenfeld *et al.*, 1985b). These observations clearly show a marked difference in accessibility and exposure of teichoic acid at the two wall surfaces. Although concanavalin A does not form a fuzzy layer at the inner wall surface, ferritin-labeled concanavalin A is seen to bind to the inner surface of isolated walls of *B. subtilis* (Beveridge and Murray, 1976), as do phages (Archibald, 1976). The phage-binding profile of walls isolated from bacilli following pulsed incorporation of a "layer" of teichoic acid (Anderson *et al.*, 1978b; Archibald, 1985) shows that teichoic acid chains are accessible to phages at the inner wall surface for only a short time after incorporation and are quickly covered up by teichuronic acid-peptidoglycan complex made after teichoic synthesis has stopped. As the teichoic acid moves into the middle layer of the wall, it becomes obscured from phages at either surface but then moves into the outer region of the wall to become fully accessible.

In *B. subtilis* W23, material in the outer 40% or so of the wall forms a diffuse region in which wall material is present at a low packing density (Anderson *et al.*, 1978b). The packing density at the poles may differ from that in the cylinder since negatively charged groups are more densely distributed around the pole than elsewhere (Sonnenfeld *et al.*, 1985a).

The picture of the wall that results from these studies is one in which the outer wall region is a high-porosity, loosely packed gel that is freely permeable to macromolecules which can enter the gel and bind to wall components within the loose gel as well as at the "surface." The defined outer surface seen in stained thin section may represent collapsed or condensed material and the fuzzy layer seen with concanavalin A may represent a "glycocalyx" that contains both peptidoglycan and teichoic acid.

Wall structure and the arrangement of polymers in the wall are influenced by the osmolarity, ionic strength, and pH of the suspending liquid. In media of low ionic strength, repulsive interactions between similarly charged groups will ensure their maximum separation: teichoic acid chains will adopt rigid rod-type conformations rather than the random coil structure adopted in high salt (Doyle *et al.*, 1975), and electromechanical interactions within the gel structure of the wall will tend to increase its volume. In contrast, high salt will give a more compact structure (Mar-

quis, 1968). In some bacteria the effects of pH and salt may alter the disposition of polymers so much that different polymers may become exposed at the surface (James and Brewer, 1968). The wall, and not just the underlying protoplast, undergoes shrinkage when *B. megaterium* is plasmolysed in media of high osmolarity, emphasizing again the elastic character of wall.

3.3. Proteins

Characteristically the walls of gram-positive bacteria contain less protein than those of the gram-negative bacteria, but substantial amounts may be present in some cases. Small amounts of protein or polypeptide are very firmly associated with the wall in *B. subtilis* (Mobley *et al.*, 1983) and cannot be removed by treatment with salts, detergent, or phenol. Such treatment can, however, remove other proteins that are also quite firmly associated. Proteins associated with the wall include enzymes involved in crosslinking and modifying peptidoglycan, and both autolytic enzymes and "modifier" proteins involved in the modulation of their activity exhibit a specific binding to wall anionic polymers that is required for their function (Herbold and Glaser, 1975a; Robson and Baddiley, 1977). In general these proteins are present in relatively small amounts in the wall and over 90% of the wall isolated even under fairly gentle conditions may be composed of peptidoglycan and anionic polymers.

s-Layer Proteins

Very much larger amounts of protein are found as envelope components that form an outermost layer of wall in a number of bacteria, including several species of *Bacillus*. These surface layers (s layers) are composed of protein or glycoprotein subunits that are arranged in hexagonal or tetragonal arrays over the whole surface of the cell (Sleytr, 1983). Though their specific function is not clear, these semi-crystalline sheets of protein form the interface between the cell and the environment, and so are responsible for the surface properties of the bacteria. They act as receptors for phages and they will presumably afford some protection to the underlying wall, possibly acting as a barrier to macromolecules (Sleytr *et al.*, 1986). S layers may also act like the outer membrane in gram-negative bacteria in trapping potentially exocellular enzymes in a surface location where they can destroy potentially damaging or toxic components from the environment.

The presence of fine structure on the wall of a bacillus was first reported by Lablaw and Mosley (1954), who examined an unidentified gram-positive bacterium. Subsequently, a regularly spaced protein layer was observed in *B. polymyxa* NCIB 4747 (Goundry *et al.*, 1967; Nermut and Murray, 1967). This forms a square lattice of tetrameric units which could be washed off the wall by SDS or urea to give sheets of a structured material that could self-assemble on its own, or onto an appropriate surface such as protein-free wall. The s-layer protein is associated with carbohydrate and it constitutes about 85% of the weight of the wall, the remainder being the underlying peptidoglycan.

Surface layers have subsequently been observed in several strains of *Bacillus*, with tetragonal and with hexagonal arrays (Sleytr, 1983). Among the best studied

are the s layer in *B. sphaericus* NTCC 9602 composed of an acidic glycoprotein containing a single homogeneous peptide chain and the s layer in *B. brevis* 47. *Bacillus brevis* has a three-layered wall, between 27 and 29 nm thick, in which the thin innermost layer of peptidoglycan and teichoic acid is covered by hexagonal array of subunits with a center-to-center spacing of 14.5 nm and composed of a 150-kDa protein (Tsuboi *et al.*, 1982). On top of this is a second s layer composed of a serologically distinct 130-kDa protein. These proteins are shed during growth (Yamada *et al.*, 1981; Ohmizu *et al.*, 1983). Udaoka and his colleagues carried out detailed studies on optimization of this secretion which can result in accumulation of up to 12 g of extracellular protein per liter of culture (Miyashiro *et al.*, 1980). Foreign proteins can also be exported with high efficiency and *B. stearothermophilus* α -amylase was secreted much more efficiently from *B. brevis* than from other hosts that carried the gene. *Bacillus brevis* may thus be useful as a host for the production of heterologous proteins (Tsukagoshi *et al.*, 1985).

S-layer proteins in rod-shaped bacteria appear to cover the surface completely to give lattices that are well ordered and fairly uniform over the cylindrical surface but which have numerous faults (dislocations and disinclinations) at the poles and septation sites that arise because of their geometry. Analysis of the distribution of faults suggests that the constituent subunits are incorporated at specific sites and recrystallize during cell growth maintaining a lowest-energy-state equilibrium (Sleytr, 1983). The s layer thus provides an ordered protein meshwork that requires a minimum of genetic information to ensure that it encloses the growing cell.

4. BIOSYNTHESIS, ASSEMBLY, AND TURNOVER OF THE WALL

Synthesis of the component polymers of the cell wall takes place in different locations within the cell. First the nucleotide precursors are synthesized by cytoplasmic enzymes, some of which may be loosely associated with the membrane (Hancock and Baddiley, 1985). In the next stage the various units are transferred from the nucleotides to undecaprenol phosphate, which acts as a lipid carrier in the membrane. Finally the units are polymerized by membrane-bound enzyme complexes and then linked into the wall by transpeptidases active at the wall-membrane interface.

4.1. Peptidoglycan

Biosynthesis of peptidoglycan proceeds by polymerization of disaccharide-peptide subunits that are assembled on a lipid carrier to give a substituted glycan in which *N*-acetylmuramic acid is present at the reducing (or potentially reducing) terminal end of the chain. Phospho-*N*-acetylmuramylpentapeptide is transferred to undecaprenol phosphate by a membrane-bound translocase to give undecaprenol pyrophosphate *N*-acetylmuramylpentapeptide (UDP) and uridine monophosphate (UMP). *N*-Acetylglucosamine is then transferred from UDP *N*-acetylglucosamine to form the disaccharide-pentapeptide-lipid *N*-acetylglucosaminyl- β -(1,4)-*N*-acetylmuramyl(pentapeptide)-*P,P*-undecaprenol. This lipid-linked disaccharide pentapeptide is used directly for peptidoglycan synthesis in some bacteria but in various

species of *Bacillus* it is first amidated at the free carboxyl group of either glutamic acid or diaminopimelic acid.

Polymerization of the disaccharide pentapeptide subunits takes place with both the growing glycan chain and the incoming disaccharide pentapeptide unit linked to carrier lipid. Studies with membrane preparations from *B. licheniformis* (Ward and Perkins, 1973) and *B. megaterium* (Fuchs-Cleveland and Gilvarg, 1976) show that new units are incorporated at the lipid-linked end of the growing glycan chain so that the nascent chain is transferred to the incoming disaccharide unit and not *vice versa*. Transfer of the growing chain releases undecaprenol pyrophosphate, which is then hydrolyzed to the monophosphate which can accept a new unit from its nucleotide. Thus the membrane-bound enzyme complex can accommodate the lipid anchors of both the growing chain and the incoming disaccharide.

The "biosynthetic" chain length of the glycan is about 100 disaccharide units in *B. subtilis* 168 and about 140 disaccharide units in a nonlytic mutant of *B. licheniformis* (Ward, 1973) but these chains are subsequently hydrolyzed and about equal amounts of both *N*-acetylmuramic acid and *N*-acetylglucosamine occur at the reducing terminus of glycan chains in walls of *B. subtilis* and *B. licheniformis*.

Postsynthetic hydrolysis of the glycan can also be effected by a *N*-acetylase. This can remove the acetyl groups from up to 70% of the *N*-acetylglucosamine residues in *B. cereus* (Araki *et al.*, 1980); de-*N*-acetylation makes the peptidoglycan resistant to egg-white lysozyme but, interestingly, more sensitive to the bacterial autolytic enzyme system. About 35% of the glucosamine in *B. subtilis* AHU 103 is present in *N*-unsubstituted form (Yoneyama *et al.*, 1984) as are 88% of the glucosamine residues and 34% of the muramic acid residues in *B. anthracis* (Zipperle *et al.*, 1984). As a result the walls are initially resistant to lysozyme but they become sensitive after chemical *N*-acetylation.

Isolated enzyme systems, reverting protoplasts, and bacteria in the presence of penicillin make only uncrosslinked peptidoglycan, but in growing bacteria the chains of peptidoglycan become linked to the wall by transpeptidases acting at the wall-membrane interface. These form crosslinkages through transpeptidation reactions in which, at least in *Bacillus*, the D-alanyl-D-alanine terminus of the donor peptide side chain of the nascent peptidoglycan is cleaved and a new peptide link is made between the remaining D-alanine and a free amino group in an acceptor peptide in the wall (Ward and Perkins, 1974; Giles and Reynolds, 1979).

4.2. Anionic Polymers

4.2.1. Wall Teichoic Acid

Studies on *B. cereus* (Muruzumi *et al.*, 1981), *B. subtilis* (McArthur *et al.*, 1981; Yokoyama *et al.*, 1986), and other bacteria show that the first stage in the assembly of wall teichoic acid is the transfer of *N*-acetylaminosugars from nucleotides to lipid carrier which then accepts glycerol phosphate units from CDP-glycerol to give the complete linkage unit. This then acts as acceptor in synthesis of the main chain of the teichoic acid by successive transfers of glycerol or ribitol phosphate units from their nucleotides. The teichoic acid chain thus extends by addition to the end remote from the lipid carrier (Kennedy and Shaw, 1968). This direction of chain extension is opposite to that of peptidoglycan.

Incorporation of sugar substituents probably takes place simultaneously with chain extension in growing bacteria, even for teichoic acids in which the sugar substituent does not form an integral part of the polymer chain.

The mechanism of incorporation of D-alanyl substituents has proved difficult to elucidate but recent work (Section 2.3) suggests that alanyl substituents may become incorporated into wall teichoic acid by transalanylation from lipoteichoic acid.

4.2.2. Teichuronic Acids

Particulate enzyme systems from *B. megaterium* synthesize teichuronic acid by successive transfer of *N*-acetylglucosamine, *N*-acetylmannosaminuronic acid, and glucose from their uridine nucleotides to a lipid carrier that is probably undecaprenol pyrophosphate. The resulting trisaccharide intermediate forms the repeating unit of the teichuronic acid "backbone" and is polymerized while the growing chain remains in pyrophosphate linkage to the carrier lipid (Arakawa and Ito, 1986).

Biosynthesis of teichuronic acid has also been studied in *B. licheniformis* (Ward and Curtis, 1982). *N*-Acetylgalactosamine-1-phosphate is transferred from its uridine nucleotide to a lipid carrier, presumed to be isoprenylphosphate. Subsequent transfer of glucuronic acid gives a disaccharide-lipid intermediate. This acts as acceptor of the polymer chain which is transferred from its lipid carrier. The growing chain thus remains linked to lipid carrier and the direction of chain extension is similar to that of peptidoglycan and the *O*-antigenic chain of lipopolysaccharide (LPS). New units are incorporated at the lipid end of the chain so that the membrane site can accommodate the lipid anchors of both the growing chain and the incoming unit. The similarity in mechanism of teichuronic acid and peptidoglycan biosynthesis has the consequence that both are inhibited by bacitracin, which blocks the dephosphorylation of the undecaprenol pyrophosphate liberated after incorporation of each new disaccharide unit.

Neutral polysaccharides, present in addition to teichoic and teichuronic acid in walls of some *Bacillus* strains, are also synthesized on undecaprenol pyrophosphate (Murazumi *et al.*, 1986).

4.2.3. Incorporation of Anionic Polymers into the Wall

In growing bacteria newly made teichoic and teichuronic acid molecules become attached only to newly made peptidoglycan (Mauck and Glaser, 1972). The peptidoglycan must apparently be undergoing crosslinkage into the wall since the presence of penicillin, which blocks the formation of crosslinkages, results in the synthesis of soluble peptidoglycan which is not attached to anionic polymers (Tynecka and Ward, 1975). Similar observations have been made with wall membrane preparations from *B. licheniformis* which synthesize teichoic or teichuronic acids from their nucleotide precursors but attach these polymers to the wall only if peptidoglycan synthesis is proceeding at the same time. Penicillin does not inhibit synthesis of the polymers but blocks their attachment to peptidoglycan (Wyke and Ward, 1977b).

The apparent requirement for ongoing synthesis of crosslinked peptidoglycan

may reflect a requirement for a particular spatial organization of the biosynthetic enzyme system and the nascent wall that is necessary for crosslinking and formation of linkage between anionic polymer and peptidoglycan. Thus some wall membrane preparations from *B. subtilis* W23 (Wyke and Ward, 1977a) are able to link new teichoic acid to preexisting wall as are toluene-treated whole cells (Hancock, 1981).

The precise location in the membrane at which the linkage between teichoic acid and peptidoglycan is formed is not known, nor indeed is the precise location at which the subunits of peptidoglycan and teichoic acid are polymerized. Since the nucleotide precursors of wall polymers are made in the cytoplasm, one possibility is that polymerization reactions occur at or near the inner (cytoplasmic) surface of the membrane and the growing polymer chains are then extruded through the membrane, pushed through by the incoming new units. Teichoic acid could not be extruded in quite this way since the growing end of the chain is that remote from the lipid anchor; both ends must presumably remain associated with membrane throughout synthesis. It could be imagined that teichoic acid becomes linked to peptidoglycan, and so detached from its lipid anchor, while new units are still being added to its growing end: the nascent teichoic acid would, in effect, be pulled out with peptidoglycan. However, this mechanism could not be used for teichuronic acid, which grows in the opposite direction to teichoic acid, nor would it explain how penicillin blocks attachment of teichoic acid and peptidoglycan.

A much more likely possibility is that the polymerization occurs on the exterior face of the membrane. This would require transport of lipid intermediates through the membrane and return of the lipid carrier to be recharged with new units at the inner surface. It is thought to be unlikely that transmembrane diffusion or "flip-flop" of lipid-linked intermediates could occur at anything near a high enough rate to support wall synthesis in growing bacteria (McCloskey and Troy, 1980), but there is nonetheless good experimental support for the presence of polymerase activity at the external surface of the membrane. Thus cells of *B. subtilis* W23 that have undergone partial wall autolysis will synthesise peptidoglycan and teichoic acid from externally added nucleotide precursors: they do not transport the nucleotides through the membrane and the polymers are formed externally (Harrington and Baddiley, 1983). This suggests that the synthetic sites are located at the outer surface of the membrane or that a transmembrane biosynthetic complex becomes temporarily exposed at the outer surface at some stage of the synthetic cycle.

Baddiley has suggested that a "rotating" or "reorienting" transmembrane complex is responsible for the uptake of precursors at the inner surface of the membrane and the formation of polymers at the outer surface (Bertram *et al.*, 1981; Baddiley, 1985). It is possible that the presence of polymerase activity at the external surface is due to damage or reorganization of the membrane even under the very gentle conditions used to achieve partial wall autolysis, though this in itself would indicate a tendency for the synthetic sites to reorient themselves in the membrane. Tentative support for the idea of a rotating or reorienting transmembrane complex has come from the observation (Harrington and Baddiley, 1984) that *in vivo* synthesis of teichoic acid and peptidoglycan, but not of their nucleotide precursors nor of lipids or lipoteichoic acid, is inhibited by valinomycin. The inhibition appears to be due specifically to its effect on the electrochemical proton gradient, maintenance of which could be necessary to provide energy for the translocation.

4.3. Organization and Location of Wall-Synthesizing Sites

The enzymes responsible for the synthesis of wall polymers seem to be present in tightly organized complexes in which a common pool of lipid carrier is used for the coordinated synthesis of both peptidoglycan and anionic polymers (Watkinson *et al.*, 1971). Individual complexes may contain enzymes for synthesis of only one kind of anionic polymer so that individual teichoic acid chains in walls of *B. subtilis* W23 (Chin *et al.*, 1966) are either fully glucosylated or else devoid of glucosyl substituents. Likewise individual peptidoglycan chains in *B. licheniformis* are attached exclusively to either teichoic acid or teichuronic acid (Ward and Curtis, 1982). The product of the activities of the biosynthetic complexes is thus "wall material" consisting of both peptidoglycan and an anionic polymer. Though most consideration to date has been focused on the incorporation of peptidoglycan into the wall, the associated anionic polymers are incorporated simultaneously and have an influence on the assembly process. Mutant bacilli that are conditionally blocked in anionic polymer synthesis exhibit gross morphological disturbances when grown under nonpermissive conditions (Boylan and Mendelson, 1969; Forsberg *et al.*, 1973). The normal assembly processes thus act to incorporate wall material in which the presence of both peptidoglycan and anionic polymer is important.

The biosynthetic complexes are located in "domains" in the membrane that can readily be separated after fragmentation of the membrane from other domains that are involved in protein translocation (Caufield *et al.*, 1983). The wall-synthesizing domains may also contain the anchoring sites for DNA (Bone *et al.*, 1985) so that they constitute distinct regions of the membrane responsible for the coordination of wall synthesis, and chromosome replication and segregation. So far, information on the location of these domains is indirect and somewhat ambiguous. Observations on wall assembly patterns in bacilli (de Chastelier *et al.*, 1975; Anderson *et al.*, 1978b; Mobley *et al.*, 1984) suggest that active biosynthetic complexes are concentrated at the developing septum and along the cylindrical length of the membrane but absent round the poles. New wall material appears to become incorporated along all of the inner surface of the cylinder (Archibald, 1976) but not at the old poles. Diminished synthetic activity at the poles is consistent with the observation (Shohayeb and Chopra, 1985) that membrane from minicells of *B. subtilis* synthesizes peptidoglycan at only 5% of the rate of whole-cell membrane: minicell membrane also differs in its phospholipid composition and in its content of penicillin-binding proteins and dehydrogenases.

4.4. Cell Wall Assembly

At least two distinguishable processes—cylinder elongation and septation—are involved in cell wall assembly in bacilli. Earlier models (see Sargent, 1978), in which new wall is incorporated from a single annular growth zone located centrally, or near a cell pole, and responsible for both septation and elongation are not supported experimentally.

Septation proceeds from an annular growth zone that lays down a disk of wall material, bisecting the cell, that is then cleaved by autolysins so forming new polar caps. The even cleavage of the septum during cell separation may be explained (Kirchner *et al.*, 1984) on the basis (Joliffe *et al.*, 1981) that autolysins are regulated

by the energized state of the membrane so that they are inactive in the vicinity of energized membrane. This would explain turnover from only the outer region of the wall (see Section 4.5) and also bisection of the septum down a plane equidistant from the energized membranes of the daughter cells. The change in shape following cleavage of the septum is due to the effect of turgor pressure, and lysis can give rise to new polar caps of normal morphology even in the absence of further wall synthesis (Koch and Burdett, 1986a,b).

Although septum formation proceeds from an annular zone of synthesis, elongation proceeds differently and by a process that involves the incorporation of new material along all of the inner surface of the cylindrical region of the wall (Fan *et al.*, 1975; Archibald, 1976). During growth this layer of new material is underlaid by yet newer material until eventually it reaches the outer surface of the wall. Only then does it become accessible to surface markers such as phages and lectins (Archibald and Coapes, 1976; Anderson *et al.*, 1978b; Mobley *et al.*, 1984) and susceptible to removal by turnover (Pooley, 1976a,b).

Fan and his colleagues (1975) suggested that during its passage through the wall a layer becomes progressively "stretched" as the cell grows, so accounting for the apparent tension in the outer wall layer that is indicated by the way in which isolated wall fragments curl up. Supporting evidence that wall material occupies a greater area when it reaches the outer surface was obtained by considering its accessibility to autolysins (Pooley 1976a) and, directly, by the observation that phages could bind to the whole of the outer surface of bacilli which had ceased synthesis of receptor material more than a generation time previously (Archibald and Coapes, 1976). Pooley suggested that the process by which wall material occupied an increasing area following its incorporation was one of "spreading." Anderson *et al.* (1978b) commented that such spreading could involve rearrangement of wall material by transpeptidation reactions involving wall of different ages or that it could be explained on the basis that wall material is present at a lower packing density in the outer wall region, as would result from the stretching proposed by Fan and colleagues.

Koch (1983) pointed out that stretching should make the peptidoglycan more susceptible to the action of autolysins and he proposes that wall growth is driven by turgor pressure. One of the principal functions of the wall is, of course, to resist this pressure and prevent the cell from bursting due to the unrestricted inflow of water. Koch suggests that the strain will be taken by stretched peptidoglycan in the middle and outer regions of the wall while new peptidoglycan can be incorporated in an unstressed state at the inner surface. Growth proceeds by a "cutting" of the stretched peptidoglycan in the middle region of the wall so that it expands further. The strain is then taken by underlying peptidoglycan while the outer layers of peptidoglycan undergo progressive stretching and cutting as they move outward to the surface from where they are removed as soluble turnover products (Koch and Doyle, 1985).

Although turnover removes old wall material from the cylindrical region of the wall, polar wall material is conserved for several generation times (Archibald and Coapes, 1976; Sturman and Archibald, 1978; Mobley *et al.*, 1984). Koch (1983) proposed that the poles act as fixed supports to permit cylindrical elongation by the stretch and cut mechanism. That mechanism alone would not explain what happens at the junction between conserved and nonconserved regions where cylinder

wall material appears to be covalently attached to polar material differing in age by several generation times. It is difficult to account for this without invoking remodeling reactions like those suggested earlier to explain cylindrical elongation and it does not seem possible to exclude such reactions as part of the wall assembly process.

The conclusion that wall material becomes stressed only some time after its incorporation is supported by the studies of Mendelson and his colleagues on filamentous mutants of *B. subtilis*. These grow as tightly twisted helices that super-fold to form macrofibers (Mendelson, 1976, 1978, 1982a). Twisting, like the writhing movements seen during growth, reflects the disposition of peptidoglycan in the wall and can range from tight right-handed to tight left-handed according to growth conditions (Mendelson *et al.*, 1984). Live macrofibers exposed to lysozyme undergo a sequence of relaxation motions showing that the structures are under tension and that peptidoglycan is involved in maintaining the stress. Changes in growth conditions can reverse the direction of twist (Mendelson and Karamata, 1982), though only after an interval that corresponds to the time taken for new wall material to move from the inner to the outer region of the wall (Favre *et al.*, 1985). The twist due to the anisotropic arrangement of the peptidoglycan is thus observed only when that peptidoglycan is in the older, i.e., the stressed, region of the wall.

Mendelson (1982a) suggested that the twisting seen in the filamentous mutants can be explained on the basis that wall material is incorporated as fibers or "strings" wrapped helically with respect to the long axis. As layers of such fibers move outward through the wall, they will come to bear the stress and become stretched out so that the pitch angle increases. Incorporation of helical fibers round the inner surface of the wall cylinder would suggest the presence of a limited number of organized synthetic sites which would presumably either extrude fibers that wind round the inner surface of the cylinder or themselves move in a helical path around the long axis of the cell incorporating wall material as they progress.

Mendelson (1982b, 1985) also suggested that this change in pitch angle might constitute a "clock" for the timing of cell cycle events and, further, that chromosome segregation might be effected through attachment to a wall string, the ends of which move apart as the pitch angle increases during growth. That segregation of the chromosomes in growing bacilli might involve their attachment to surface structures additional to the membrane was first suggested by Eberle and Lark (1966) and is supported by numerous observations showing disordered growth and division in protoplasts and wall-less L forms. Segregation of the chromosome attachment sites through wall growth has been a feature of the various of zonal growth models that have been proposed (see Sargent, 1978). The finding (Archibald and Coapes, 1976) that only the cell poles are conserved excluded most of these models but raised the possibility that the poles might be implicated in anchoring and segregating the chromosomes. Both origin and terminus regions of the chromosomes are associated with the cell wall in *B. subtilis* (Doyle *et al.*, 1983; Sonnenfeld *et al.*, 1985c) and a model has been suggested (Koch *et al.*, 1981) in which specific association with the cell poles participates in the coordination of segregation and septation. In itself, of course, segregation of such chromosome attachment sites would not serve to separate the chromosomes given that the total length of the latter is many times greater than that of the whole cells. DNA can move in cells independently of length extension (Mendelson, 1972) and it has been suggested that the release of termini leads to

a jump from their original sites to new sites associated with daughter origins (Sargent, 1975). The forces responsible are not yet understood and the mechanisms controlling the coordinated segregation of daughter genomes and the cell division that leads to their compartmentalization in separate cells remains to be elucidated.

4.5. Autolysins and Cell Wall Turnover

Strikingly rapid autolysis can be observed when cultures of *Bacillus* are incubated under conditions that are unfavorable for growth. This is due to hydrolysis of peptidoglycan by autolysins present in the wall and is observed under conditions such as oxygen deprivation or exhaustion of the energy source that lead to deactivation of the membrane. Uncoupling agents also promote autolysis showing that control of the autolytic enzyme system is effected by energized membrane (Joliffe *et al.*, 1981).

Autolysins act by depolymerizing peptidoglycan and do this by hydrolyzing either the glycan or the crosslinked peptides, or the linkages between them. Glycan hydrolysis can be effected by either *N*-acetylmuramidase or *N*-acetylglucosaminidase activities, both of which have been reported in *Bacillus* though the latter is more common. A variety of different peptidases has been found in bacteria but in *Bacillus* the most common autolysin is the *N*-acetylmuramyl-L-alanine amidase that splits the linkage between glycan and peptide. This amidase occurs together with glucosaminidase in several strains of *B. subtilis* from which both have been purified after extraction from isolated walls by treatment with salt (Herbold and Glaser, 1975b; Rogers *et al.*, 1984). Isolated β -*N*-acetylglucosaminidase from *B. subtilis* 168 exists as a dimer with a subunit molecular mass of 90,000 and pH optimum about 5.0: the amidase is a relatively small monomeric enzyme of molecular mass 30,000–50,000. The enzymes are regulated differently. The glycanase is activated by low concentration of Li^+ and inhibited by acylated lipoteichoic acid and phospholipids, whereas the amidase is not inhibited by these compounds but is activated by Mg^{2+} .

Differences in molecular mass and pH optima have been reported for autolysins from different strains of *Bacillus* (see Rogers *et al.*, 1980) even though they hydrolyse similar linkages. Autolysins generally show high affinity for homologous walls and several examples are known where binding to an anionic polymer in the wall is a prerequisite for hydrolysis of peptidoglycan. In *B. licheniformis*, for example, the autolysin is held in the wall by binding to teichuronic acid and the *nov-12* mutant, which is deficient in teichuronic acid, is resistant to autolysin with the consequence that it grows in long, multiseptate filaments (Robson and Baddiley, 1977). The ability of isolated amidase to bind to walls of *B. subtilis* is diminished when teichoic acid is removed and subtle effects on autolysin activity are also observed due to the allosteric “modifier” subunit that binds to teichoic acid and affects both the extent and the nature of amidase activity (Herbold and Glaser, 1975a).

Mutants that lack or are resistant to autolysins grow in long, multiseptate filaments (Mendelson, 1976; Tilby, 1977), showing a role for these enzymes in cell separation. Autolytic enzymes are involved in the development of competence (Young *et al.*, 1967). They appear to be involved in the bactericidal, though not the bacteriostatic, effect of β -lactam antibiotics and in sporulation and germination (see Rogers *et al.*, 1980). It has also been suggested that they may function in the

extrusion of flagella (Fein, 1979), though alternative explanations of the relationship between autolysin and flagellum production have been proposed (Pooley and Karamata, 1984).

One consequence of the activity of autolysins in bacilli is the shedding of wall material during growth. This process of wall turnover involves the removal of old wall material (Mauck *et al.*, 1971) from the outer surface layers of the wall (Pooley, 1976a,b). It has been suggested that turnover may constitute part of the normal process of cylindrical extension (Glaser and Lindsay, 1977; Koch and Doyle, 1985), though mutants are known that show greatly reduced turnover, and the principal effect of diminished autolysin activity is the formation of chains of unseparated cells in which, nevertheless, the component cells seem to be of normal length.

Given their potential to destroy the cell, the autolytic enzymes must be under tight control in growing bacteria. The ionic environment can substantially affect autolysin activity (Cheung and Freese, 1985) as can secreted proteases (Joliffe *et al.*, 1980). Lipoteichoic acid (Section 2.3) can also inhibit autolysins though their participation in this has not been demonstrated in growing cells for which the most important overall regulatory mechanism seems to be associated with energized membrane (Joliffe *et al.*, 1981).

The precise location of hydrolytic cleavage may depend on the stress on the peptidoglycan and its distance from the membrane, and also on the location and distribution of autolysins within the wall so that, for example, the diminished turnover at the polar caps may be due to a lower content of autolysin at the poles (Clarke-Sturman and Archibald, 1981). The kinetics of turnover and wall polymer synthesis are discussed in Section 7.

4.6. Regeneration of Protoplasts

Under appropriate conditions enzymic removal of the cell wall from *Bacillus* yields protoplasts which can remain viable and grow as L forms although growth is irregular and division is disturbed (Landman *et al.*, 1977). Under appropriate conditions protoplasts can regenerate to the walled form when growth again becomes regular.

There is increasing interest in the use of protoplasts for the genetic analysis and manipulation of *Bacillus* species by protoplast-mediated transformation (Chang and Cohen, 1979; McDonald and Burke, 1984) and protoplast fusion techniques (Schaeffer *et al.*, 1976; Schaeffer and Hirschbein, 1985).

Procedures for the formation of protoplasts generally involve digestion with egg-white lysozyme in osmotically protecting media containing divalent cations. Following transformation, the protoplasts are allowed to regenerate by incubation on agar plates containing complex media and additives such as gelatin (see Chapter 4, Section 2).

Regeneration is affected by the presence of autolysins and can be enhanced by addition of cell wall preparations or of proteases to absorb or inactivate them (Landman *et al.*, 1977). Conditions favoring regeneration of protoplasts from several different species of *Bacillus* have been described by Bourne and Dancer (1986). Detailed biochemical and morphological studies of regeneration have been described in *B. licheniformis* (Elliott *et al.*, 1975a,b). Fibrils of wall material gradually

surround the protoplast to form a fringe up to 400 nm thick. This gradually becomes more compact though the regenerating cells continue to show abnormal septation and other gross morphological abnormalities, and a more normal rod shape and division pattern is established only slowly.

5. EFFECT OF GROWTH CONDITIONS ON ENVELOPE COMPOSITION AND SURFACE PROPERTIES

The cell wall in *Bacillus* is a dynamically changing structure (Ellwood and Tempest, 1972) in which old material is constantly being replaced by newer material in turnover and assembly processes that continuously modify existing wall material over the whole of the cell, though less rapidly at the poles than elsewhere. In bacteria growing under balanced conditions this continuous modification involves wall material of differing age, but in bacteria undergoing changes in growth conditions the composition as well as the age of wall material is changed and rapid alterations to wall and surface properties can result.

The composition of the protoplast membrane is also markedly affected by growth conditions. *Bacillus cereus*, *B. subtilis*, and *B. stearothermophilus* maintain membrane fluidity on growth at low temperature by increasing their content of the anteiso series of fatty acids (Boudreaux and Freese, 1981). The lipid composition of *B. subtilis* can be varied, and greatly simplified, by manipulation of medium composition. Thus phosphate-limited cells contain a greatly reduced proportion of phosphatidylglycerol compared to bacteria grown in excess of phosphate (Minnikin *et al.*, 1972). Phosphate limitation has also been reported to reduce the lipoteichoic acid content of *B. licheniformis* to 10% of that present in bacteria grown under other limitations (Button *et al.*, 1975). Membrane proteins also differ in bacteria grown under different conditions. Some of these differences are due to changes in wall-synthesizing enzyme systems affecting the anionic polymers (Thompson *et al.*, 1980).

Though not well documented in *Bacillus* species, phenotypic alterations in peptidoglycan structure have been described in other bacteria which can involve substantial differences in the extent of crosslinking and susceptibility to enzymic hydrolysis (see Archibald and Heckels, 1975). To date most attention to the effect of growth conditions on walls in bacilli has been given to the remarkable change that is observed when the bacteria are grown under conditions of phosphate limitation. Teichoic acid, which can account for up to half the weight of the wall in bacteria growing under other conditions, is completely replaced by teichuronic acid (Ellwood and Tempest, 1969, 1972). Phosphate-limited cells of *B. subtilis* 168 contain only one-third as much phosphorus as is present in cells grown in ample phosphate (Lang *et al.*, 1982) and the ability to replace teichoic acid by teichuronic acid permits the bacteria to use the limited phosphate efficiently for only the more essential cellular phosphates, such as nucleic acids.

When grown under mixed limitation, where a second nutrient is present in potentially limiting amounts, *B. subtilis* makes teichoic acid in amounts that reflect the amount of phosphate present over and above that needed to sustain "phosphate-limited" growth (Anderson *et al.*, 1978a). The bacteria can be grown under balanced conditions so that they contain varied amounts of teichoic and teichuronic

acids, the precise amounts of which can be tailored by adjusting the medium composition. The total anionic polymer content (teichoic acid plus teichuronic acid) remains nearly constant but bacilli that contain differing proportions of the two anionic polymers differ in their electrophoretic mobility-pH profiles. They also differ in the speed with which they agglutinate at low pH values (I. C. Hancock, personal communication) and in their ability to bind phages and lectins (Givan *et al.*, 1982; Lang and Archibald, 1983).

Less well explored than the variation in the type of anionic polymer is the variation in the proportion of alanylation in the teichoic acid. An early study (Ellwood and Tempest, 1972) showed that the proportion of alanine ester in walls of *B. subtilis* and *B. licheniformis* varies according to growth conditions. It is well established that alanyl groups affect the properties of teichoic acids. The amino group of the alanine neutralizes the adjacent phosphate and so determines its ability to bind cations (Heptinstall *et al.*, 1970). The "compactness" of the wall is also affected by the presence of alanyl groups and removal of alanine can result in substantial increases in its dextran impermeable volume. The ability of lipoteichoic acid (LTA) to inhibit autolysin also depends on the alanine content (Fischer *et al.*, 1981), and the inactivity of highly alanylated lipoteichoic acid suggests that neutralization of the phosphate charges may block interaction between LTA and autolysin. Binding of proteins to teichoic acid may generally be affected by the presence or absence of alanine, although no evidence has been given.

Studies on the regulation of anionic polymer synthesis show that the amount of teichoic acid incorporated into walls of *Bacillus* growing under such differing steady-state phosphate concentrations is controlled by changes in the activity of enzymes catalyzing the synthesis of the lipid-linked "linkage unit" (Cheah *et al.*, 1982; Hancock, 1983, 1985). The activity of enzymes effecting synthesis of the teichoic acid main chain did not vary with varied phosphate and decreased only when phosphate became fully limiting. The site of regulation of teichoic acid synthesis is thus the lipid-linked linkage unit. An increasing body of evidence (see Hancock, 1985) suggests that in general wall composition is regulated by the interaction of individual synthetic pathways at the level of enzymic reactions involving the formation of intermediates linked to the isoprenyl phosphate carrier lipid.

The effects of growth conditions on wall chemistry could have several consequences. For example, phage contamination has caused serious damage to fermentation productivity in Japanese industry in a variety of processes including the production of amino acids, antibiotics, dairy products, and solvents (Ogata, 1980). Infections have occurred in amylase-producing strains *B. amyloliquefasciens* and in the production of natto (fermented soy beans) by *B. subtilis* var. *natto* where phage infection results in reduced product viscosity. Serious problems have been encountered in the production of bacitracin due to phage infection in the producing organism (*B. licheniformis*), although these can be overcome by the adoption of a variety of preventive measures including improvement of the overall cleanliness of the environment, better sterilization procedures, and the use of phage-resistant mutants. Another approach has been to add chemical agents to halt the multiplication and spread of infection. For example, many phages need divalent cations (Ca^{2+} , Mg^{2+}) for adsorption to the wall and injection of DNA, and chelating agents have been used in attempts to block this process. Teichoic acids form an essential part of the receptor for many *B. subtilis* phages (see Archibald, 1980) and, since

adsorption requires appropriate wall chemistry, susceptibility to infection and the protection afforded by chelating agents may be affected by differences in wall composition resulting from growth conditions.

Since growth conditions can profoundly affect envelope composition and surface properties, it follows that consideration has to be given to the effect of growth conditions not only on the efficiency of growth or product formation but also on downstream processing operations that involve agglutination, sedimentation, filtration, disruption, or enzymic hydrolysis.

6. ROLE OF ENVELOPE COMPONENTS IN PROTEIN SECRETION

Bacillus strains are of major importance for the production of enzymes used in food and other industries. The ability of members of this genus to export large amounts of protein (see Section 3.3) suggests that they may be well suited as agents for the expression of heterologous genes and the secretion of their products.

Most work on protein export in *Bacillus* has concerned the process of secretion through the cytoplasmic membrane (see Randall and Hardy, 1984). Nucleotide sequence analysis of genes encoding secreted proteins in *B. subtilis* shows N-terminal signal sequences similar to those found in gram-negative species, though an additional sequence between those of the signal and the mature exoprotein is found in many cases and may be involved in interactions with the membrane (Nielson *et al.*, 1981).

Little attention has been paid to possible interactions with wall components, or to the process by which exported proteins pass through the wall into the culture fluid, but the efficiency with which gram-positive bacteria secrete proteins is thought to reflect the absence of the outer membrane type of structure that traps exported proteins in the periplasmic gel region in gram-negative bacteria. Nevertheless, the wall in *Bacillus* may not be freely permeable to all proteins and it is possible that both the porosity of the wall and the affinity of wall components for proteins may affect secretion.

Translation and secretion may be coupled and may involve both wall and membrane components. A limiting factor in protein export seems to be the capacity of the secretory machinery, which can become saturated (Sibakov *et al.*, 1983) and also monopolized by one particular protein to the exclusion of others. Thus the pleiotropic *sacU^h* mutants of *B. subtilis* show changes in both protein export and envelope structure (Chambert and Petit-Glatron, 1984). The mutants secrete 100 times as much levansucrase as the similarly induced parent strain but secrete other proteins in greatly diminished amounts, suggesting that the mutation has resulted in an enhanced affinity between the levansucrase and the secretory machinery. The lesion also results in the development of a *Lyt*⁻ phenotype so that the *sacU^h* mutants lack flagella, are nontransformable, and grow in long filaments.

It is reasonable to suppose that the altered envelope properties and protein secretion are related. Chambert and Petit-Glatron (1984) suggested that both could be explained on the basis of an increased production of an envelope component, perhaps lipoteichoic acid, that could inhibit autolytic enzymes and also form a complex with levansucrase that would have high affinity for the secretory machine-

ry. It may, however, be unnecessary to postulate an inhibitor of autolytic activity since any change in the secretory machinery that results in its activity being directed exclusively to export of levansucrase would inevitably diminish the autolytic capacity of the bacteria because autolytic enzymes would be among the proteins whose export ceased. The primary effect of the lesion could therefore be on protein export, the *Lyt*⁻ phenotype reflecting impaired export of autolysin.

While the role suggested for lipoteichoic acid in levansucrase production is purely speculative, affinity between proteins and wall components is well documented (see Section 3.3). Several "exported" proteins appear to remain firmly but noncovalently held to bacterial cell surface structures, presumably by ionic and other interactions with envelope components, but there have been apparently contradictory reports about the location of a number of exported enzymes. For example, alkaline phosphatase in *B. licheniformis* has been reported in a secreted form present in the culture supernatant (Chesbro and Lampen, 1968), in a cell-bound form released by lysozyme (Glynn *et al.*, 1977), and in salt-extractable and detergent-extractable membrane forms (Hulett *et al.*, 1976; Schaffel and Hulett, 1978).

The detergent-extractable enzyme is located on the inner leaflet of the cytoplasmic membrane (McNicholas and Hulett, 1977) and is immunochemically similar to the other forms, although it may possibly be distinguished genetically since recent work (Hulett *et al.*, 1985) showed that *B. licheniformis* contains two alkaline phosphatase genes that code for similar proteins but are controlled by different promoters.

The composition of the cytoplasmic membrane may have a substantial effect on the secretion and localization of enzymes (Petit-Glatron and Chambert, 1981) and the precise location and distribution of the secreted form of alkaline phosphatase seems to depend on growth conditions, differences in which can result in the enzyme being almost wholly retained in the envelope or almost wholly secreted into the culture fluid. These differences may be due to effects of growth conditions on the chemistry of the envelope and to the effect of different ionic conditions on the strength of interactions between proteins and envelope components. Thus high pH and salt both release alkaline phosphatase from *B. licheniformis* (Wouters and Buijsman, 1977).

Release of protein from the surface to the surrounding fluid may be seen as the final stage in the secretory process but the processes by which proteins pass from the outer surface of the membrane to the outer surface of the cell are not well studied. While it may be possible for small proteins to diffuse rapidly outward through the wall, bigger proteins and proteins that are capable of binding to wall components may not have unhindered egress. Studies on intact bacteria have shown that the wall is not permeable to concanavalin A (Lang and Archibald, 1983). Williamson and Ward (1981) showed that treatment of intact *Bacillus* with a solution of 5 M LiCl extracted proteins with a molecular mass up to 70 kDa, which might thus represent the maximum porosity of the wall, at least to proteins in concentrated lithium chloride. In contrast, physical studies (Scherrer and Gerhardt, 1971) suggest that walls are unlikely to be freely permeable to molecules having a radius of more than 1.1 nm, corresponding to a molecular mass of about 1200.

Direct evidence that the wall is a barrier to secretion was obtained by Gould and his colleagues (Gould *et al.*, 1975), who suggested that proteins are secreted through

the membrane into a pool that then diffuses slowly through the wall. A delay in secretion into the medium has also been reported (Archibald, 1985) in more recent experiments in which chemostat cultures of *B. subtilis* were pulse-labeled with radiolabeled amino acids. It was suggested that part of the secretory machinery for some proteins might involve the normal wall assembly process in *Bacillus*, in which new wall material is incorporated at the inner wall surface of the wall and then pushed outward to the outer surface during growth (see Section 4.4). Proteins could be picked up by wall material, either by physical entrapment because of their size or by chemical association because of specific or nonspecific affinity, and carried with it through the wall to the surface to be released under appropriate ionic conditions.

It is possible that many proteins may indeed simply diffuse through the wall, though these would be expected to be smaller proteins and not affected by the ionic character of the wall, and that more specialized mechanisms (such as "pores" created by autolysins) may be involved in some cases. The topic would benefit from further study but the available information suggests that the whole envelope, and the effect of growth conditions on its structure and composition, are important determinants in protein export and secretion into culture fluid.

7. KINETICS OF WALL SYNTHESIS AND TURNOVER

The cell wall in *Bacillus* is a dynamically and phenotypically varying structure. Earlier sections described the mechanisms of wall assembly and the changes in wall composition that result from changes in growth conditions. This section considers the rate at which these processes occur.

7.1. Rate of Synthesis of Wall Polymers

In bacteria growing under balanced or steady-state conditions, the rate constant for synthesis of all cellular components is the same, and is equal to the specific growth rate μ . Thus:

$$\frac{dB}{dt} = \mu B \quad (1)$$

and

$$\frac{dW}{dt} = \mu W \quad (2)$$

where B and W are the amounts of whole bacteria and intact cell wall. In *Bacillus* wall material is shed by turnover and the total amount of cell wall material present is the sum of that present in the cells and in extracellular turnover product. During balanced growth both the cell wall and the turnover product will increase at the growth rate so that the ratio (a) of turnover product (T) to intact cell wall (W) will be constant (Pooley, 1976a). Provided that there is no direct secretion of wall polymers

from the bacteria, the total amount of wall material present (WM) will be the sum of that (W) present in the bacteria and that ($T = aW$) present in the culture fluid, i.e.,

$$\begin{aligned} WM &= W + aW \\ &= (1 + a)W \end{aligned} \quad (3)$$

where WM , W , and a are as defined above.

So, although the rate of increase of cell wall is that given by Eq. (2), the rate of synthesis of cell wall material is given by

$$\begin{aligned} \frac{dWM}{dT} &= \mu WM \\ &= \mu(1 + a)W \end{aligned} \quad (4)$$

Consequently, because of wall turnover, more cell wall material is made than would be expected simply from the rate of growth of the cell wall. The rate of wall polymer synthesis may be determined by both the bacterial growth rate and the extent of wall turnover so that the greater the turnover, i.e., the greater the value of a in Eq. (4), the greater will be the amount of wall material synthesized. Alternatively, it is possible that changes in turnover caused by mutation or by changes in growth conditions do not affect the rate of wall polymer synthesis but are instead accompanied by changes in the proportion of the total wall polymer present in the intact cell wall. The cellular wall content would thus represent the balance between synthesis and turnover of wall material.

7.2. Rate of Incorporation of New Material into the Wall

In wild-type bacilli turnover product can constitute more than a third of the total wall material present so that substantially more wall polymer is synthesized than would be necessary simply to provide material for new intact wall. New wall material is, however, not susceptible to removal by turnover until a considerable time, designated t^* , after it is incorporated. The value of t^* can be between three-quarters and two generation times (Mauck *et al.*, 1971; Pooley, 1976a). Since turnover involves removal of only older wall material, all of the newly synthesized wall material remains in the wall. A consequence of this is that the walls contain a greater than expected proportion of "new" material, and so changes in wall composition that result from changed growth conditions can occur rapidly (Ellwood and Tempest, 1969; Archibald and Coapes, 1976). The amount of new material in the wall can be deduced as shown. During balanced growth wall increases as in (2) so that, by integration,

$$W = W_0 e^{\mu t} \quad (5)$$

The rate of synthesis of wall material shown in (4) can therefore be written as

$$\frac{dWM}{dT} = \mu(1 + a)W_0 e^{\mu t} \quad (6)$$

from which the amount of wall material synthesized between zero time and time t is

$$WM_t - WM_0 = W_0(I + a)(e^{\mu t} - 1) \quad (6)$$

When $t < t^*$ all of this new material will be present in the intact cell wall which thus contains $(1 + a)$ times as much new wall as would be expected simply on the basis of (2).

For $t > t^*$, the proportion of old and new material present in the wall can be calculated from the above and wall turnover kinetics using equations such as those described by de Boer *et al.* (1981) and Koch and Doyle (1985).

7.3. Kinetics of Wall Turnover

Pooley (1976a) showed that cell wall material in *B. subtilis* is initially resistant to turnover but, after a delay, becomes susceptible to removal by a process that follows first-order kinetics. Pooley proposed that turnover involved removal of material from only the outer region of the wall and a mathematical model of this was described (de Boer *et al.*, 1981). This model was extended by Koch and Doyle (1985) who carried out computer simulations. These mechanistic models have been used to show that the mechanism of wall growth in bacilli results in certain relationships between growth and turnover, e.g., that the turnover rate constant and the growth rate constant will normally have the same value. The validity of these relationships remains to be established and the kinetics can be described mathematically without reference to any particular "mechanistic" model and in a way that does not impose restrictions on possible turnover rates. Such an analysis (Archibald, 1987) can be used to describe wall metabolism and composition changes, and gives a satisfactory description of what is observed, particularly if allowance is made for the fraction of the wall (concentrated in the poles) that is resistant to turnover.

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Fermentation and Downstream Processing of *Bacillus*

10

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1. INTRODUCTION

The genus *Bacillus* comprises a heterogeneous group of chemoorganotrophic, aerobic, rod-shaped microorganisms. These include both mesophilic and thermophilic species as well as acidophiles and alkalophiles. One of their main characteristics is their ability to produce heat-resistant endospores.

The genus contains a number of industrially important species. Apart from the *B. cereus* group, including *B. anthracis*, they are saprophytic producing no toxins and are not regarded as pathogenic to man. *Bacillus subtilis* has GRAS (generally regarded as safe) clearance in the United States. They are able to secrete a number of enzymes and are important commercial sources of proteolytic and amylolytic enzymes (Priest, 1977; see Chapter 11). Other commercially important processes include the production of glucose isomerase, a number of polypeptide antibiotics, and insecticidal toxins. More recently, they have become important hosts for the expression of recombinant DNA for the production of a range of proteins. *Bacillus* species are generally easy to grow to high cell density and do not require expensive growth factors.

There is considerable information available on laboratory scale fermentation of *Bacillus*, but as processes become more commercially significant, the availability of published literature declines. Therefore the information available on large-scale industrial practices becomes generalized, with competitors being unwilling to disclose the more significant and novel details of their processes.

The greatest improvements in the productivity of fermentations are generally ascribed as due to improvements in the microorganism following mutation and strain selection studies. However, the nutritional and physical environment used to cultivate the organism and the methods used for product recovery and purification

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also have a significant effect on the economics and final product yield of the process.

The media used to isolate and screen industrial microorganisms are not usually those used for development studies and production. These latter media often include components which are relatively expensive, support suboptimal growth, or may be regarded as undesirable for use at the production scale. Development of an economical process requires the selection of a source of carbon, nitrogen, phosphorus, sulfur, potassium, and trace elements. An energy source is required which will maximize microbial growth and product yield, with the maximum yield of product or biomass per unit of substrate. The rate of product formation must be maximized and undesirable side products kept to a minimum. The ingredients must be cheap, of consistent quality, and readily available all year, and the costs of transport and any pretreatment should be minimal. Industrial media are generally based on the use of natural materials, although a careful balance of carbohydrate, nitrogen, and mineral salts is essential. The use of natural materials which are generally agricultural commodity products results in some geographic variation in availability, composition, and cost, which should be considered when carrying out media formulation studies.

While industrial fermentation media are based on complex nutrients, development studies require the use of empirical procedures to assess the suitability of medium ingredients for growth, and for product induction and repression. Medium development is generally initiated at the level of the shake flask, but pilot scale fermentation studies generally indicate further modifications. Statistical methods for medium optimization are discussed by Greasham and Inamine (1986).

Since sporulation terminates cell metabolism, it is often desirable to select and use asporogenous mutants; spores may also survive the recovery process resulting in microbial contamination during downstream processing. The production of polypeptide antibiotics by *Bacillus* species has been widely exploited, but their occurrence as secondary products in a process where the primary product is intended for use in the food or pharmaceutical industry is undesirable. Selection of suitable non-antibiotic-producing mutants is therefore necessary in these cases.

2. EXTRACELLULAR ENZYMES

Extracellular enzymes are generally regarded as those enzymes which cross the cytoplasmic membrane. In general, extracellular enzymes are hydrolases able to depolymerize complex macromolecules, including proteins, polysaccharides, and nucleic acids. Exceptions include β -lactamases which hydrolyze the lactam ring of penicillin. Extracellular enzymes are produced by a wide group of bacteria including *Bacillus*, *Clostridium*, *Cytophaga*, *Actinomyces*, and some *Pseudomonas* species. The commercial exploitation of extracellular enzymes from *Bacillus* strains was initiated some 70 years ago with the production of α -amylase from cells grown as surface cultures in semisolid medium. The ability of strains to secrete enzymes presents considerable advantages for product recovery. The problems of cell breakage and the removal of viscous nucleic acids associated with recovery of intracellular enzymes are avoided. Enzyme productivity may also be enhanced since the yield is not restricted by the level of biomass produced.

The key factor which determines whether an enzyme is to remain intracellular or be secreted is the presence of a hydrophobic NH_2 terminal signal sequence composed of some 15–30 amino acids. This signal peptide appears during translation to direct the ribosome to the membrane where polypeptide is then translocated through the membrane. Translocation appears to require the presence of membrane-bound proteins, although their role is not yet clearly understood (Holland *et al.*, 1986). The signal sequence is cleaved by signal peptidases during translocation. Studies to maximize the expression of cloned foreign proteins have led to expression levels where the desired protein comprises 30–60% of the total cellular protein. High levels of expression of intracellular proteins can cause the product to form inclusion bodies in which the protein may be irreversibly denatured. The fusion of genes encoding intracellular enzymes to sequences encoding the signal peptides of exoenzymes leads to the secretion of the enzyme from the cell. This avoids the problems of inclusion body formation and may enhance product recovery. Problems of proteolysis of the product may occur when the enzyme is secreted, although the isolation of protease-deficient *B. subtilis* mutants have been reported.

The production of extracellular enzymes is controlled by catabolite repression, enabling microorganisms to utilize rapidly catabolizable carbon sources in preference to more complex substrates present in the environment. α -Amylase synthesis from *B. licheniformis* and *B. amyloliquefaciens* is repressed in the presence of glucose (Ingle and Boyer, 1976; Priest and Thirunavukkarasu, 1985) while proteases from *B. licheniformis*, *B. amyloliquefaciens*, and *B. subtilis* are repressed in the presence of readily metabolized amino acids (Ingle and Boyer, 1976). Problems of catabolite repression may be overcome by using complex medium ingredients and by the selection of strains in which catabolite repression has been overcome. (Priest and Sharp, 1988).

The production of extracellular enzymes is a relatively simple process. Microorganisms are cultured in a suitable medium and the enzyme subsequently recovered from the medium, after removal of the cells. The problems for production center on achieving a process which is economical and reliable and complies with the strict codes for safety and production laid down by the regulatory authorities such as the Food and Drug Administration (FDA) in the USA or the Medicines Inspectorate or Health and Safety Executive (HSE) in the United Kingdom. Process plant and equipment must be maintained in good order and a record kept of production and quality control data.

2.1. α -Amylases

The value of worldwide sales for α -amylase in 1985 was in the region of £14.8 million. Bacterial α -amylases are used for the liquefaction of starch to allow the efficient production of dextrose by saccharifying enzymes (see Chapter 11). They are endo-amylases capable of random hydrolysis of 1,4- α -glucosidic linkages of amylose, amylopectin, and glycogen. The hydrolysis of amylose results in complete conversion to maltose and maltotriose, followed by a second-stage reaction which slowly hydrolyzes maltotriose to glucose and maltose. Hydrolysis of amylopectin yields glucose and maltose as well as a series of β -limit dextrans comprising branched oligosaccharides of four or more glucose units containing the 1,6- α -glucosidic linkages of the original polymer (Fogarty and Kelly, 1979). α -Amylases are produced by

a wide group of microorganisms although commercial production has centered on *B. amyloliquefaciens* and, more recently, *B. licheniformis*. The enzyme is widely used in the food industry for the production of syrups and in the brewing industry for the liquefaction of starchy materials used as adjuncts prior to enzyme saccharification. The textile industry utilizes the enzyme for the removal of starch-based sizes used for reinforcing fibers during weaving, and the paper industry uses the enzyme in the preparation of starch-based adhesives.

2.1.1. Strain Selection

α -Amylases have been isolated from a wide range of microorganisms, including many species of *Bacillus*. The preliminary isolation of strains able to hydrolyze starch is carried out using conventional plating onto solid medium using starch as the primary carbon source. Colonies showing evidence of starch hydrolysis can then be detected by flooding the plate with iodine to detect zones of hydrolysis (Priest, 1984). *Bacillus* strains able to hydrolyze starch have been isolated from soil, compost, ground litter, and aerobic sludge processes. Thermophilic, acidiphilic, and alkaliphilic strains of *Bacillus* producing α -amylase have been described (Sharp and Munster, 1986). Following the selection of a potential α -amylase producer, strain development to enhance production may follow either the conventional mutation and selection procedures or may involve genetic manipulation. The results of industrial strain development strategies are rarely published. Bailey and Markkanen (1975) described a strain improvement program in which they utilized a number of mutagenic agents including ultraviolet light irradiation, ethyleneimine, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine to obtain a twofold improvement in α -amylase production from *B. subtilis*. The use of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was reported to give a two- to threefold increase in α -amylase production from a strain of *B. subtilis* (Marburg) (Yoneda and Maruo, 1975).

2.1.2. Medium Development

Fermentation of α -amylase-producing *Bacillus* strains is based on naturally occurring materials such as maize, wheat, malt, soy extracts, and corn steep liquor (Table I). Fukumoto *et al.* (1957) described a medium for the production of α -amylase from *B. amyloliquefaciens* based on the following: soya bean cake extracts (6%), potato starch (3.5%) (dextrinized with bacterial amylase), $(\text{NH}_4)_2$ (1.2%), KCl and MgSO_4 (0.02%). Studies of amylase production from *B. subtilis* indicated that when carbohydrate was used as the carbon source, phosphate had a significant effect on enzyme production. The optimum phosphate concentration was in the region of 0.1 M. When casein and soya bean extracts were used as nitrogen source, the choice of carbon source was not critical; starch, most sugars, and organic acids were all suitable. Peptones and amino acids provided a more effective source of nitrogen than ammonium.

A 300-fold increase in α -amylase production from *B. amyloliquefaciens* (ATCC 23350) was achieved when glycine was added to a chemically defined medium (Zhang *et al.*, 1983). Studies by Fukumoto *et al.* (1957) using washed cells of *B. subtilis* (probably *B. amyloliquefaciens*) in a phosphate buffered medium with 1%

soluble starch showed glycine- and cysteine-inhibited enzyme production. The concentration of Fe^{3+} ions was critical to enzyme production (Coleman and Elliott, 1962). Di-ammonium hydrogen phosphate was the preferred source of nitrogen and phosphate for α -amylase production from "*B. apiaris*" CBML-152 (Ghosh and Chandra, 1984). Enzyme production was enhanced by Mn^{2+} , Ca^{2+} , and Zn^{2+} although there was little effect on cell growth; Mg^{2+} , Fe^{2+} , Fe^{3+} , and Na^{+} enhanced both growth and enzyme production. Nine amino acids (alanine, arginine, asparagine, aspartic acid, cystine, histidine, phenylalanine, lysine, and valine) stimulated growth and enzyme production when examined individually in a starch-based mineral salts medium. A combination of arginine, cystine, lysine, and valine were found to be the most effective in stimulating enzyme production and their omission resulted in a decrease in activity. Biotin, pantothenic acid, pyridoxine, and thiamine all enhanced growth and enzyme production. Studies with naturally occurring substrates indicated that using oil seed cakes and husks of pulses enhanced production. Dried and powdered mushrooms also increased production in the presence or absence of peptone and yeast extract. α -Amylase production from *B. licheniformis* CUMC-305 was supported using alanine, arginine, aspartic acid, cystine, isoleucine, phenylalanine, and serine. The omission of aspartic acid and cystine resulted in a 50% reduction in enzyme production. Biotin, pantothenic acid, and thiamine enhanced production; $(\text{NH}_4)_2\text{PO}_4$ and peptone were the optimum source of phosphate and nitrogen. Galactose, dextrin, glycogen, and inulin were the optimum sources of carbon; low concentrations (0.05–0.1%) of glucose, lactose, maltose, fructose, rhamnose, and sorbitol supported moderate production, but higher concentrations were inhibitory. Na^{2+} , Cu^{2+} , Mn^{2+} , Mg^{2+} , and Fe^{3+} all stimulated enzyme production (Chandra *et al.*, 1980). The use of oil seed cakes in a basal medium containing peptone and beef extract were found to provide an ideal fermentation base for the production of amylase from this strain of *B. licheniformis* (Krishnan and Chandra, 1982). The use of sesame, linseed, and cottonseed cakes required the addition of corn steep liquor to provide an additional nitrogen source.

A medium for the cultivation of *B. licheniformis* (ATCC 39326) was based on 10% lactose as carbon source, with K_2HPO_4 (81.4%), KH_2PO_4 (0.6%), $(\text{NH}_4)_2\text{SO}_4$ (0.6%), sodium citrate (0.3%), whole-grain soya flour (3%), and $\text{CaCl}_2(2\text{H}_2\text{O})$ (0.05%). Alternative carbon sources included glucose, starch, or complex carbohydrate-containing materials such as corn meal. The optimum carbohydrate concentration was considered to be 5–15% (w/v). The nitrogen source was based on soluble nitrates or ammonium salts or organic sources such as yeast extract, corn steep liquor, or peanut meal (Horwath, 1984).

The production of a thermostable amylase from *B. stearothermophilus* (strain 1503-4) was examined in a defined medium with 0.1% casein hydrolyzate (Welker and Campbell, 1963a). The enzyme appeared constitutive since enzyme activity was observed on glycerol, glucose, and sucrose; addition of casein hydrolyzate (0.1%) permitted growth on fructose. Growth did not occur on sodium acetate or sodium succinate. Maximum production was observed on starch and maltose which induced higher levels of amylase. Glycerol, glucose, and sucrose exhibited an inverse relationship between the rate of growth and total amylase production, indicative of catabolite repression. Analysis of technical grade maltose indicated the presence of 3–5% glucose, 0.5% maltotriose, and 2.5% of higher molecular weight maltodextrins. Studies with maltodextrins as individual constituents showed maximum enzyme induction with maltotetraose, followed by maltopentaose, maltohexaose, malt-

otriose, and maltose (Welker and Campbell, 1963b). A soil isolate of *B. stearothermophilus* with a lower optimum growth temperature than the strain studied by Welker and Campbell (1963a) produced an amylase with an optimum temperature for activity of 82°C (Srivastava and Baruah, 1986). Maximal production was observed on a complex medium based on 0.4% corn steep liquor; moderate production was found with meat extracts. Growth was comparable on yeast extract but enzyme levels decreased. Starch was required to induce amylase synthesis, and maximum enzyme production was observed in the late exponential and stationary phases of growth. Isoleucine, phenylalanine, cysteine, and aspartic acid were essential for enzyme production and glutamic acid, methionine, and glycine were stimulatory. Di-ammonium hydrogen phosphate and ammonium dihydrogen phosphate were preferable to ammonium sulfate and ammonium nitrate as nitrogen sources. Ca^{2+} , Sr^{2+} , Mg^{2+} , and Ba^{2+} slightly stimulated enzyme production whereas Ag^{2+} , Cu^{2+} , Fe^{3+} , Sb^{3+} , As^{3+} , and Au^{3+} inhibited enzyme production and An^{2+} and Mn^{2+} had no effect. Growth on glucose, galactose, fructose, and sucrose suppressed enzyme production. Enzyme activity was not observed on maltose; however, amylase was induced with maltotriose, maltotetraose, maltopentaose, and maltohexaose. Maximum production was observed on starch, dextrin, and glycogen and the disaccharide cellobiose.

Thermostable amylase was extracted from the culture supernatant of 10 strains of *B. stearothermophilus* isolated from heat-treated aerobic sewage sludge (Grueninger *et al.*, 1984). In a basal salts medium of pH 7.0 supplemented with malt extract, yeast extract, and simple carbon sources, growth of all 10 strains was observed. Amylase activity was only produced on media containing either lactose (six strains), acetate (three strains), or maltose (two strains), no activity was observed on glucose, fructose, galactose, sucrose, glycerol, and citrate. Growth on soy grist, rape grist, and molasses increased amylase production by 10- to 50-fold compared with growth on simple carbon sources. Enzyme yields were lower on peptone, casein, wheat flour, or corn steep liquors and no activity was found in supernatants of cells grown on soluble or insoluble starch. Combinations of complex substrates gave increased enzyme yields and the best combination examined was malt extract/soy grist/peptone. Studies appeared to indicate that low growth rate, which could be achieved by decreasing agitation or aeration, enhances amylase production.

Buonocore *et al.* (1976) studied the effect of different carbon sources on amylase production from *B. acidocaldarius* using a basal salts medium supplemented with 0.5% yeast extract and 0.5% carbon source. Enzyme production occurred during the stationary phase of growth in the presence of oligo- or polysaccharides having 1,4- α -glucosidic linkage. Maximum enzyme yields were achieved after 18 hr using glycogen, although starch and maltose also produced high enzyme activity. No enzyme activity was detected following growth on glucose, glycerol, lactose, sucrose, trehalose, or cellobiose. Amylase production increased linearly with inducer concentration up to a maximum of 0.3–0.5%; above this concentration enzyme activity slowly declined. There was no significant variation in the cell growth when the inducer was varied.

2.1.3. Fermentation Studies

Laboratory and pilot scale fermentation studies of amylase production for a number of different species of *Bacillus* have been reported. These have, in the

main, involved the use of small-batch and continuous-fermentation studies to evaluate the control of enzyme production, optimization of media, and physical growth parameters.

Continuous culture studies of amylase and protease synthesis by *B. amyloliquefaciens* and some strains of *B. subtilis* have, after initially showing high enzyme levels in batch culture, often been followed by fluctuating enzyme yields which subsequently decline during continuous culture (Heineken and O'Connor, 1972; Fencel *et al.*, 1972; Sikyta and Fencel, 1976). This pattern of enzyme yield can be explained by the occurrence of low-producing or nonproducing mutants (Sikyta and Fencel, 1976; Fencel *et al.*, 1979). The regulation of synthesis of extracellular enzymes in these two organisms also provides an explanation. In *B. subtilis* Marburg and *B. amyloliquefaciens*, extracellular enzyme synthesis is repressed during exponential growth and depressed during early stationary phase prior to sporulation. Since the chemostat provides continuous exponential growth, extracellular enzyme synthesis would be repressed. Fencel and Pazlarova (1982) studied α -amylase production from *B. subtilis* using a two-stage chemostat in which the enzyme concentration in the second stage reached more than 75% of the maximum concentration achieved in batch fermentation.

Strains of *B. stearothermophilus* (Davis *et al.*, 1980), *B. licheniformis* (Emanuilova and Toda, 1984; Priest and Thirunavukkarasu, 1985), and *B. polymyxa* (Griffin and Fogarty, 1973) in which amylase production occurs during exponential growth all exhibit constant levels of amylase production during growth in continuous culture.

Chemostat studies of *B. licheniformis*, grown under nitrogen limitation with glucose, glucose and glutamate, or glucose and alanine as carbon sources, indicated minimal production of α -amylase. Growth under carbon limitation, with glucose, alanine, or glutamate as carbon source, showed that amylase production was optimal at a dilution rate (D) of 0.05/hr and decreased by more than 50% as D was increased to 0.2/hr. Although low growth rate resulted in an increased rate of enzyme production per gram of substrate, the rate of enzyme production per unit fermentation volume was independent of dilution rate (Meers, 1972). In batch fermentation studies using soluble starch as carbon source, a range of polymers from glucose to maltohexaose was detected. Within 10 hr of inoculation, the sugar concentration decreased to a level at which it was no longer detectable and α -amylase production was initiated. Increasing the concentration of glucose significantly diminished the rate of amylase production until the glucose was dissimilated. Glucose produced in the fermenter as a result of α -amylase activity was metabolized and did not accumulate to detectable levels. Repression of α -amylase activity was also observed with fructose and maltose.

Catabolite repression was observed in *B. caldolyticus* (Emanuilova and Toda, 1984), *B. stearothermophilus* (Srivastava and Mathur, 1984), and *B. acidocaldarius* (Buonocore *et al.*, 1976). Studies in batch cultures of *B. licheniformis* (NCIB 6346) also indicate catabolite repression by glucose (Priest and Thirunavukkarasu, 1985). In the chemostat under maltose limitation, amylase production was maximal at $D = 0.24$ /hr and declined at higher growth rates. Optimum production was observed above pH 7.5 and decreased rapidly below pH 6.5. Enzyme production was constitutive; the growth rate was higher on maltose and maltotriose than on xylose or arabinose, although amylase secretion on maltose and maltotriose was reduced during early exponential growth possibly due to catabolite repression. α -Amylase

synthesis was therefore initiated at a high cell density and it accumulated more rapidly than in the slower growing cultures on xylose and arabinose. The final yield of α -amylase was similar irrespective of the carbon source.

Davis *et al.* (1980) studied α -amylase production from a strain of *B. stearothersmophilus* in a 1-liter fermentation vessel with the air flow at 1.5 vvm and the impeller speed at 300 rpm. Studies were carried out on a tryptone/maltose-based medium at at 55°C. In batch cultures the pH increased to 8.2 after 3.5 hr, yielding a maximum of 790 U of α -amylase per ml of culture. When the pH was controlled at pH 7.2, amylase concentration reached 470 U/ml after 3.5 hr, although the biomass yield was the same. In a chemostat under tryptone limitation at 55°C the α -amylase concentration increased from 513 U/ml at $D = 0.31/\text{hr}$ to 5788 U/ml at $D = 0.75/\text{hr}$. The amylase concentration remained the same up to a $D = 0.95/\text{hr}$. Above this growth rate and below 0.31/hr the concentration of amylase was greatly reduced. Cell dry weight and optical density followed a similar trend. Maximum rates of both α -amylase and biomass production were reported at a $D = 0.95/\text{hr}$. Studies of protease and α -amylase production from *B. stearothersmophilus* strains NCIB 8924 and NRRL-B-3880, growing in a medium based on beef extract, tryptone, and maltose, were examined in 10- and 300-liter fermenters (Sidler and Zuber, 1977). In a 300-liter batch fermenter with the pH controlled at 6.8 with 4 N NaOH, impeller speed at 200 rpm, air flow at .05 vvm with 0.5 atm over pressure, and at a temperature of 53°C, the cultivation time was 5–7 hr. The vessel was given a 5% inoculation from a preculture grown under similar conditions.

Emanuilova and Toda (1984) studied α -amylase production from "*B. caldolyticus*" during batch and continuous fermentation using a 2-liter fermentation vessel with a working volume of 1.5 liter. Growth temperature was maintained at 60°C, air flow at 0.5 vvm, and impeller speed at 500 rpm. The pH was not controlled, dissolved oxygen was maintained above 1.0 ppm, and foaming was controlled by use of KM70 antifoam agent. A basal salts medium with casitone (2 g/liter) was supplemented with either glucose or starch (1 or 0.1 g/liter). In batch culture with starch (1 g/liter) the pH decreased from 7.0 to 5.5 and then increased at the end of exponential phase, eventually reaching pH 8.8 in late stationary phase. Amylase production occurred during exponential growth when the cell doubling time was approximately 15 min. Without glucose or starch as carbon source, α -amylase production was minimal on casitone; addition of glucose (1 g/liter) resulted in amylase production after utilization of the glucose (approximately 3 hr). Addition also of starch (0.1 g/liter) led to immediate production of amylase with final yields similar to yields on glucose alone. Enzyme yields were doubled when casitone and starch (2 g/liter) were used in the absence of glucose (Table II).

In continuous culture amylase activity was low (3 U/ml) at dilution rates above 1.2 liter/hr when cell dry weight, glucose concentration (0.1 g/liter), and pH (5.5) remained relatively constant. The maximum specific growth rate, determined by washout experiments, was 2.7/hr. Peak amylase activity was observed at a dilution rate of 0.1–0.25/hr, one-tenth of the maximum specific growth rate. At this growth rate the concentration of glucose and starch were so low that accurate determinations were not possible. The addition of a small amount of starch had a more significant effect on amylase production in continuous cultures than in batch culture. Addition of starch (0.1 g/liter) to the casitone and glucose (1.0 g/liter) medium resulted in a 17-fold increase in productivity compared with batch

Table II. α -Amylase Production in Batch and Continuous Cultures of *B. caldolyticus*^a

Carbon source			Batch culture		Continuous culture		
Casitone (g/liter)	Glucose (g/liter)	Starch (g/liter)	Amylase (U/ml)	Amylase (U/g dry weight)	Dilution rate (h ⁻¹)	Amylase (U/ml)	Amylase (U/g dry weight)
2.0	0	0	2.5	3.0	0.1–0.9	1.5	3.2
2.0	1.0	0	9.0	9.7	0.15	5.0	7.1
2.0	1.0	0.1	8.5	7.2	0.15	80.0	120.0
2.0	0	1.0	13.5	18.0	0.25	200.0	230.0

^aData from Emanuilova and Toda, 1984.

cultures. When casitone and starch (1.0 g/liter) was used in the absence of glucose, enzyme yields increased some 15-fold over the batch fermentation, giving a yield of 200 U/ml of culture.

2.1.4. Industrial Production of α -Amylase

The industrial production of α -amylase from *B. amyloliquefaciens* was developed by Boidin and Effront (1917). The organism, which was initially named "*B. mesentericus*," was reclassified first as *B. subtilis* and later as *B. amyloliquefaciens*. *Bacillus amyloliquefaciens* is used for much of the commercial production of α -amylase. The enzyme has a molecular mass of about 50,000 and is stable over a pH range of 5.5–8.0 with optimum activity at pH 5.9. The enzyme is stabilized by calcium ions which are generally added to reaction mixtures. The enzyme can be used to hydrolyze starch at temperatures approaching 90°C. Starch granules are resistant to α -amylase attack and must first be ruptured by heat before hydrolysis can occur. "Cooking" temperatures above 100°C are normally required to ensure complete breakage of maize granules and gelatinization of the starch. Enzymes able to operate at higher temperatures would enable faster, more efficient, and cheaper starch liquefaction since the material would not have to be cooled after gelatinization for enzymic hydrolysis. α -Amylase from *B. licheniformis*, which was introduced in 1972, hydrolyzes starch at 105–110°C. The enzyme has a wider pH range than the *B. amyloliquefaciens* enzyme and has a molecular mass of 60,000. Despite being more thermostable it is less dependent on Ca²⁺. It has the disadvantage that the reaction is more difficult to terminate by heat treatments. Depending on the strain being cultivated, fermentations are carried out at 30–40°C at pH 7.0. The pH may be maintained by the addition of CaCO₃ to the medium which has the additional advantage of increasing enzyme stability. Enzyme activity is lost if the pH falls below 6.0. α -Amylase production based on complex natural ingredients (Tables I and III) begins as the cell count approaches 10⁹–10¹⁰ cells/ml, after approximately 10–20 hr. Production continues until the utilizable carbon source is exhausted, usually 100–150 hr (Aunstrup, 1979).

Both *B. amyloliquefaciens* and *B. licheniformis* produce a serine protease and *B. amyloliquefaciens* also produces a neutral protease, β -glucanase, and hemicellulase.

Table III. Production Media for the Production of α -Amylase from *Bacillus*

Components	Amount (%)	References
Ground soya bean meal	1.85	Underkofler, 1976
Amber BYF (autolyzed brewers yeast fractions, Amber Laboratories)	1.5	
Distillers dried solubles	0.76	
N-Z amine (enzymic casein hydrolyzate, Sheffield Chemical Company)	0.65	
Lactose	4.75	
MgSO ₄	0.04	Smythe <i>et al.</i> , 1950
Hodag KG-1 antifoam (Hodag Chemical Corporation)	0.05	
Water	90.40	
Cornstarch	4.0	
Ground corn (hominy)	10.0	
Corn steep liquor	6.5	Outtrup <i>et al.</i> , 1972
Water	79.5	
Potato starch	10.0	
Ground barley	5.0	
Soya bean meal	2.0	
Sodium caseinate	1.0	
Na ₂ HPO ₄ (12H ₂ O)	81.1	

The proteases must be removed or inactivated since their presence may lead to the formation of melanin-like products during starch hydrolysis. The proteases may be destroyed by heating under conditions that minimize the inactivation of α -amylase. Adsorption of the protease to clay minerals (bentonite) or fractional precipitation may also be used. The use of protease-free mutants provides an alternative but this may then require the use of prehydrolyzed protein in the fermentation medium (Aunstrup, 1979).

Seed inocula for production fermentations are generally grown in a seed fermenter and transferred aseptically to the production vessel while the culture is still in exponential growth. Depending on the size of the production fermenter, intermediate vessels may be required; a 5% v/v inoculation is generally considered desirable. The use of complex natural ingredients may require milling or pretreatment before use and separate sterilization before transfer to the production fermenter.

Amylase production in the culture supernatant may be monitored using the methods of Welker and Campbell (1963a), Pfeuller and Elliot (1969), Emanuilova and Toda (1984), or Horwath (1984).

The cells and any insoluble medium ingredients remaining need to be removed from the fermentation broth by centrifugation or filtration. Flocculating agents may be necessary to settle the cells before clarification. The clarified broth is then concentrated by low-temperature, high-vacuum evaporation. After dilution of the liquid preparation to give a standard activity, preservatives and calcium salts may be added to aid in stabilization of the preparation.

The activities of commercial preparations vary by a factor of 100 and are adjusted to suit the required application. The production of more concentrated products leads to reduced packaging and transport costs. The most active liquid preparations may contain 2% active amylase and the most active solid preparations up to 5% active amylase. Solid preparations are obtained by further evaporation of liquid, and spray drying is used to achieve a concentrated powder. Further purification steps to achieve highly active preparations include precipitation of the enzyme with sodium or ammonium sulfate, or solvent systems based on ethanol or isopropanol. To assist recovery, the enzyme may be isolated in the presence of an inert carrier such as starch, lactose, calcium sulfate, or casein depending on the application for which the product is designated (Burbidge and Collier, 1968).

2.1.5. α -Amylase Production from Recombinant *Bacillus* Strains

The cloning of the α -amylase gene and studies of its expression in various species of *Bacillus* have been widely reported (Palva, 1982; Aiba *et al.*, 1983; Ortlepp *et al.*, 1983; Joyet *et al.*, 1984; Piggott *et al.*, 1984; Mielenz and Mickel, 1985; Aiba *et al.*, 1986; Vehmaanpera and Korhola, 1986). There are few reports, however, discussing plasmid stability and gene expression during fermentation and scale-up of laboratory studies. Nybergh (1984) studied the expression of the α -amylase gene cloned from *B. amyloliquefaciens* into *B. subtilis* using a plasmid pUB110. The recombinant strain produced a fivefold increase in α -amylase compared with the *B. amyloliquefaciens* strain and a 2500-fold increase compared with the wild-type *B. subtilis* strain. Comparative fermentation studies between the original *B. amyloliquefaciens* strain and the recombinant *B. subtilis* strain were carried out in 8-dm³, 1-m³, and 10-m³ fermenters.

Vehmaanpera *et al.* (1987) cultured *B. subtilis*, carrying the α -amylase gene from *B. amyloliquefaciens* cloned into pUB110, at the 900-liter scale. Amylase production was superior to the original industrial strain used and the plasmid remained relatively stable throughout the fermentation. The recombinant strains were maintained on L agar supplemented with 1% starch. Kanamycin (5 μ g/ml) was added to plates used for subculturing. The recombinant organism produced higher levels of α -amylase than the donor strains on L broth supplemented with starch hydrolyzate, or L broth in which casein hydrolyzate was substituted for tryptone. Growth on complex raw materials available from a grain alcohol production plant and a starch sugar plant resulted in little difference in the level of amylase produced by the original *B. amyloliquefaciens* strain and the recombinant *B. subtilis*. The availability of free amino acids in these complex medium ingredients was relatively low and the greater proteolytic activity of *B. amyloliquefaciens* was a significant advantage for growth on complex substrates. Laboratory scale studies indicated that the plasmid construct was stable for some 50 generations; however, at the semi-industrial scale, loss of expression was observed resulting from deletion of part of the α -amylase gene.

Pinches *et al.* (1985) examined the expression of the α -amylase gene from an industrially important *Bacillus* strain (Bsl) cloned on plasmid pVC102 into *B. subtilis* IA297 (Corfield *et al.*, 1983, 1984). Stocks of the recombinant organism were lyophilized and maintained at 4°C. Working stocks were subcultured on L broth agar supplemented with 2% starch and containing neomycin sulfate and chloram-

phenicol (10 µg/ml). Fermentation seed cultures were prepared by transferring a few colonies from a fresh (24-hr) plate culture to 20 ml of L broth supplemented with antibiotics and incubating at 37°C for 8–10 hr on a reciprocal shaker. Plasmid loss was determined by plating on L broth agar plates supplemented with starch (LS medium) with and without antibiotics.

Fermentations (5-liter) were carried out at 37°C with pH controlled to 7.0 with H₂SO₄ and KOH. Foaming was controlled by the addition of Dow Corning DB31 silicon emulsion. The stirrer speed was maintained at 600 rpm and sterile air was supplied at 0.4 vvm. Plasmid loss was negligible during exponential growth in L broth supplemented with either starch or glucose, becoming significant only during stationary phase. In L broth supplemented with starch, α-amylase production was growth-associated and plasmid loss, which became apparent after 50 hr, had little effect on enzyme production. Enzyme production on L broth supplemented with glucose was associated with late exponential and stationary phase of growth. Loss of plasmid toward the end of exponential phase significantly affected enzyme yields although this was significantly improved following the addition of antibiotics to the medium. Plasmid instability was associated with both structural instability resulting in deletions and total elimination of the plasmid (see Chapter 7).

The cloning of a thermostable α-amylase from *B. stearothersophilus* into *B. subtilis* on plasmid pUB110 was reported by Zeaman and McCrea (1985). Studies of the α-amylase from the recombinant strain and wild-type *B. stearothersophilus* indicated them to be identical, and animal-feeding studies were carried out to confirm the safety of the recombinant product for use in the food industry. Following the completion of toxicity trials, a petition was filed with the FDA seeking affirmation of GRAS status for the cloned enzyme.

2.2. β-Lactamase

The β-lactamases are important in clinical microbiology, where their presence confers resistance to β-lactam antibiotics, and in clinical chemistry, where they are used to inactivate β-lactam antibiotics, prior to the microbiological assay of aminoglycoside antibiotics in the serum of patients undergoing dual-antibiotic therapy for severe infections. The enzymes are also important in the antibiotic industry, where there is a need to examine potential new antibiotics for their susceptibility to known β-lactamase enzymes. In addition, the enzymes are used to inactivate samples of the drug in order that suitable sterility testing can be carried out.

The term *β-lactamase* covers a range of enzymes with varying degrees of specificity. Thus some enzymes are specific for the hydrolysis of the lactam ring in penicillins (*N*-acyl derivatives of 6-aminopenicillanic acid) (Fig. 1) while others are specific for the hydrolysis of the lactam ring in cephalosporins (*N*-acyl derivatives of 6-aminocephalosporanic acid) (Fig. 2). The term *specificity* must be treated with caution because a single enzyme will often show some activity against both types of antibiotic.

β-lactamase activity was discovered by Abraham and Chain (1940) in *E. coli*, and it has subsequently been found in a wide range of microorganisms (e.g., Citri and Pollock, 1966). The enzyme was first observed in *B. cereus* by Benedict *et al.* (1946) and has subsequently been isolated from several species of *Bacillus*: *B. cereus* (Pollock, 1950; Pollock *et al.*, 1956), *B. licheniformis* (Pollock and Richmond, 1962),

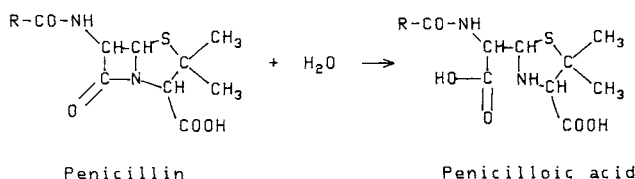


Figure 1. Hydrolysis of penicillins by β -lactamase.

B. subtilis (Ishimoto, 1963), and *B. anthracis* (Smith *et al.*, 1964). However, only the enzyme from *B. cereus* has been produced on any significant scale.

B. cereus produces two extracellular β -lactamases, termed β -lactamase I and β -lactamase II (Kuwabara, 1970). β -Lactamase I has a higher affinity for penicillins, whereas β -lactamase II is a Zn-containing enzyme and has a higher affinity for cephalosporins.

2.2.1. Strain Isolation and Development

The production of an extracellular β -lactamase by a strain of *B. cereus* deposited as NRRL 569 (NCTC 7464) was described by Benedict *et al.* (1946). The enzyme was shown by Pollock and Perret (1951) to be induced by the addition of penicillin to the medium; S broth (Difco Peptone, 10 g/liter; Bovril meat extract, 3 g/liter; NaCl, 2 g/liter). Kogut *et al.* (1956) isolated a constitutive mutant of *B. cereus* NRRL 569 (which was deposited as NCTC 9945 CNRRL 569/H). Agar containing S broth and Andrade indicator was inoculated with spores and incubated at 37°C for 16 hr. A solution of penicillin was poured over the plate and a single pink area (indicating hydrolysis of penicillin to penicilloic acid) was detected. The agar containing this colony was removed, suspended in broth, and streaked onto a fresh plate. After incubation and the addition of penicillin solution, about 30% of the colonies turned pink, showing them to be constitutive producers of penicillinase. Subsequently, this strain was further developed to one termed 569/H/9, which produces larger amounts of penicillinase than the parent constitutive strain (Davies *et al.*, 1974).

2.2.2. Fermentation and Production

The producer strain is routinely stored as a spore suspension at 4°C. Before being used for large-scale fermentation it is screened for high penicillin production

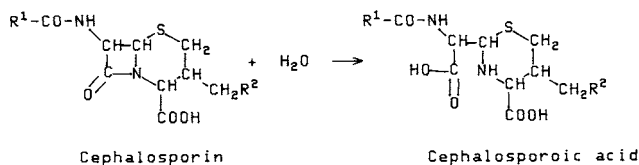


Figure 2. Hydrolysis of cephalosporins by β -lactamase.

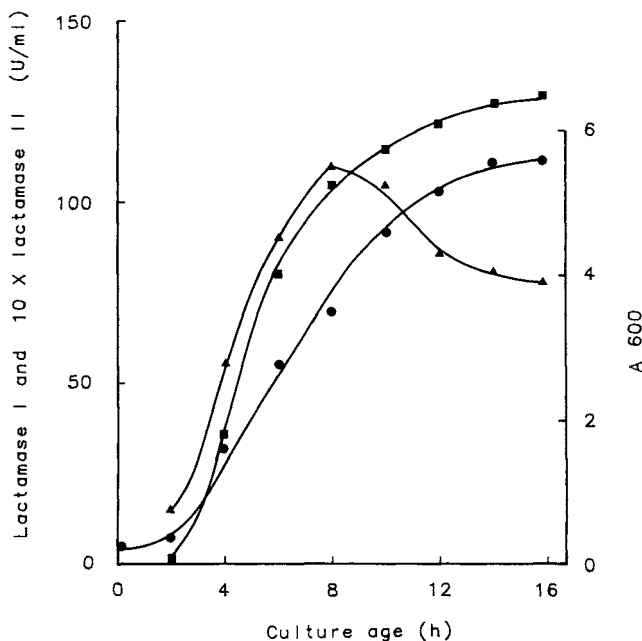


Figure 3. Production of β -lactamase I and β -lactamase II by *B. cereus* grown in 400-liter culture. (■) β -Lactamase I activity; (▲) β -lactamase II activity; (●) A_{600} .

by use of the procedure described by Kogut *et al.* (1956). The production medium comprises casamino acids, 10 g/liter; KH_2PO_4 , 2.72 g/liter; Na-citrate ($5\text{H}_2\text{O}$), 5.88 g/liter; $\text{MgSO}_4 \cdot (7\text{H}_2\text{O})$ 0.41 g/liter; $\text{FeSO}_4 \cdot (7\text{H}_2\text{O})$ 0.014 g/liter, ZnSO_4 , 0.29 g/liter, adjusted to pH 7.0 with 3 M HCl. In a 400-liter fermenter, the active culture is aerated at 300 liters/hr and stirred at 250 rpm (Davies *et al.*, 1974). The addition of ZnSO_4 was found to enhance the production of β -lactamase II by a factor of about 4 (Kuwabara, 1970).

The two enzymes are synthesized at different rates and at different stages in the culture, as shown in Fig. 3. This also shows that the amount of β -lactamase II decreases rapidly as the culture reaches its stationary phase, whereas the amount of β -lactamase I continues to increase. In order to obtain optimal amounts of both enzymes, it is important to monitor their levels and to harvest the culture at about the peak of β -lactamase II production.

2.2.3. Enzyme Recovery and Purification

It had been found, by chance, that β -lactamase I binds to powdered glass of the type used to make sintered glass filters (Kogut *et al.*, 1956), and this observation was initially exploited as a means of concentrating the enzyme from culture supernatants. However, this treatment results in large losses of β -lactamase II activity. Subsequently, the enzymes were found to bind to the diatomaceous earth, Celite 545, although again β -lactamase II was lost (Citri *et al.*, 1960; Kuwabara, 1970).

Table IV. Purification of β -Lactamases I and II from *B. cereus*^a

Step	Total protein (g)	Total enzyme (units $\times 10^{-6}$)		Specific activity (U/mg protein)	
		I	II	I	II
Culture supernatant	162	43.8	8.76	270	54
Celite	15.6	38.3	7.46	2450	480
CM Sepharose	2.46	17.7	5.84	7200	2400

^aTaken from Davies *et al.*, 1974.

However, for large-scale production the use of Celite is attractive since it is very cheap, concentrating the enzyme from dilute culture supernatants giving a useful degree of purification. More recent investigations have shown that both enzymes can be recovered with good yield from Celite, providing the adsorption is carried out at pH 7.0 rather than pH 4.5, and elution is performed with Tris-citrate buffer (pH 7.0) containing Zn^{2+} rather than in phosphate buffers at pH 8.0. The likely explanation is that β -lactamase II is less stable than β -lactamase I at pH <5, and the use of phosphate buffers may have caused a loss of Zn^{2+} from the enzyme (as insoluble zinc phosphate), leading to its irreversible inactivation.

Both enzymes have been isolated on a large scale from 400-liter cultures (Davies *et al.*, 1974) and the data are summarized in Table IV. Twenty-five kilograms of Celite was added to the culture supernatant and stirred for 30 min. The Celite was recovered by filtration, washed three times with deionized water, and packed into a column 25 cm $\times$ 37 cm diameter. After washing further with 25 liters deionized water to remove unbound proteins, the enzymes were eluted with 0.1 M Tris, 0.1 M trisodium citrate, 1 mM ZnSO_4 , and 1.5 M NaCl adjusted to pH 7.0 with 6 M HCl, and the elutant collected in 4-liter fractions. Those fractions containing β -lactamase activity were pooled, concentrated, and desalted by ultrafiltration. The two enzymes were separated and further purified by ion exchange chromatography on CM-Sephadex in 0.1 M Tris HCl, pH 7.0, and eluted with a salt gradient to 0.57 M NaCl. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the two enzymes were purified to homogeneity.

3. INTRACELLULAR ENZYMES

Although the majority of commercially important enzymes produced by strains of *Bacillus* are extracellular, numbers of the genus also produce intracellular enzymes of commercial value and some which are important for research. Perhaps the most commercially important of these is glucose isomerase, which is discussed in detail in Section 3.1. Many enzymes from *Bacillus* are used as reagents in clinical chemistry, particularly those isolated from thermophilic species which have superior stability (Atkinson, 1983; Price, 1983), e.g., glycerokinase (Comer *et al.*, 1979), glucokinase (Hengartner and Zuber, 1973; Goward *et al.*, 1986), and leucine dehydrogenase (Oshima and Soda, 1985). This same stability also accounts for the wide-

Table V. Examples of Restriction Endonucleases Produced by Bacilli

Enzyme	Source	References
<i>Bam</i> HI	<i>B. amyloliquefaciens</i>	Wilson and Young, 1975
<i>Bam</i> I	<i>B. aneurinolyticus</i>	Sugisaki <i>et al.</i> , 1982
<i>Bam</i> II	<i>B. aneurinolyticus</i>	Sugisaki <i>et al.</i> , 1982
<i>Bcl</i> I	<i>B. caldolyticus</i>	Bingham <i>et al.</i> , 1978
<i>Bgl</i> I	<i>B. globigii</i>	Duncan <i>et al.</i> , 1978
<i>Bgl</i> II	<i>B. globigii</i>	Duncan <i>et al.</i> , 1978
<i>Bst</i> I	<i>B. stearothermophilus</i>	Catterall and Welker, 1977
<i>Bst</i> EII	<i>B. stearothermophilus</i>	Lautenberger <i>et al.</i> , 1980

spread use of enzymes from thermophilic *Bacillus* strains in structural studies of enzymes, where, for example, the crystal structure of tyrosyl and tryptophanyl tRNA synthetases from *B. stearothermophilus* were determined because the enzymes from *E. coli* were insufficiently stable. *Bacillus* is also an important source of restriction endonucleases. These are produced commercially, albeit on a relatively small scale, for use as reagents in molecular genetics, as listed in Table V. The following sections give examples of the production of intracellular enzymes from strains of *Bacillus*.

3.1. Glucose Isomerase

The isomerization of glucose to fructose has considerable importance in the starch-processing industry since it enables the production of a product sweeter than sucrose and from less expensive starch hydrolyzates. There is no apparent reason why cells should require an enzyme for the conversion of glucose to fructose and in fact glucose isomerases are more accurately described as D-xylose isomerases which have D-glucose isomerase as a secondary activity. Glucose isomerase (D-xylose ketol-isomerase, EC 5.3.1.5), which catalyzes the reversible isomerization of D-glucose to D-fructose, has been isolated from a number of different species of microorganisms. The most widely used sources for commercial production are *Streptomyces* (Takasaki *et al.*, 1969) and *Bacillus coagulans* (Yoshimura *et al.*, 1966; Suekane *et al.*, 1974; Diers, 1977).

3.1.1. Strain Isolation and Development

Yoshimura *et al.* (1966) described the isolation of over 300 strains from soil and manure on a medium comprising D-xylose, 1%; yeast extract, 0.1%; K₂HPO₄, 0.05%; MgSO₄ (7H₂O), 0.025%; MnSO₄ (7H₂O), 0.001%; CaCO₃, 0.2%. Incubations were carried out for 24 hr at 45°C. Following three successive transfers to fresh medium, the isolates were plated on the above medium with 2% agar and examined for glucose isomerase activity. A strain of *B. coagulans* (HN-68) produced D-xylose- and D-glucose-isomerizing activity when grown of D-xylose as the main carbon source. The production medium comprised D-xylose, 2%; yeast extract,

0.3%; K_2HPO_4 , 0.01%; $MgSO_4$ ($7H_2O$), 0.05%; $MnSO_4$ ($7H_2O$), 0.005%; NH_4Cl , 0.3%; and $CaCO_3$, 0.2%. Maximum enzyme production occurred after 14–16 hr incubation at 40°C at pH 7.0. Glucose isomerase activity was induced in cells grown on D-glucose following exposure to D-xylose.

Outtrup (1976) described a method for isolating at high frequency organisms producing a heat-stable glucose isomerase with a low optimum pH for activity. The procedure involved the incubation of soil samples on media with ammonia as the sole nitrogen source and xylose as the sole carbon source at 60–65°C to ensure that only thermophilic strains were isolated. The isolation medium comprised $(NH_4)_2SO_4$, 0.03%; K_2HPO_4 , 0.01%; $MgSO_4$ ($7H_2O$), 0.005%; KCl, 0.005%; $FeSO_4$, 0.001%; xylose, 0.5%; agar, 2%. Colonies were subcultured to complex medium with xylose as the major carbon source and incubated at 60°C before testing for the presence of glucose isomerase. Eighteen strains of *Bacillus* were isolated, giving conversion efficiencies of 35–51%. Constitutive mutants, isolated following treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, exhibited glucose isomerase activity when grown on a yeast extract medium without xylose or glucose.

Suekane *et al.* (1974) described the isolation of strains of *B. stearothermophilus* from soil using a medium containing xylose, 0.5%; soluble starch, 0.5%; peptone, 0.5%; corn steep liquor, 1%; meat extract, 0.5%; yeast extract, 0.25%; NaCl, 0.05%; $MgSO_4$ ($7H_2O$), 0.05%; $CoCl_2$ ($6H_2O$), 0.024%; and agar, 2%. The medium was prepared at pH 7.0 and inoculated plates were incubated for a few days at 60°C. Seventeen strains with glucose isomerase activity were isolated and identified as strains of *B. stearothermophilus*.

3.1.2. Fermentation and Production of Glucose Isomerase

Diers (1976, 1977) reported batch and continuous culture studies using the constitutive mutant strain (NRRL 5650) developed by Outtrup (1976). Glucose isomerase production did not require xylose for induction, the strain grew between 37 and 65°C with an optimum of 55–60°C and at pH 4.5–8.5. For batch culture a medium based on corn steep liquor was devised comprising corn steep liquor (50% dry weight), 3%; K_2HPO_4 , 0.015%; $(NH_4)_2SO_4$, 0.5%; glucose, 0.2%; $MgSO_4$ ($7H_2O$), 0.01%; $MnSO_4$ (H_2O), 0.01%; pluronic H-61, 0.03%, pH 6.8 with 30% NaOH. In a 10-liter fermenter air was sparged into the culture at 1 vvm, the impeller speed was maintained at 620 rpm, and the temperature at 50°C. Thirteen hours after inoculation batch growth terminated and the pH began to rise as the oxygen tension reached zero. When the pH reached 6.5, 40% glucose was fed to the vessel at a rate of 2.1 g/liter/hr enabling the pH to be maintained constant for the next 8 hr. The dissolved oxygen concentration increased and growth terminated. The culture gave a cell yield of 4.2 g/liter and an enzyme yield of 8.8 U of glucose isomerase per milliliter of culture.

Diers (1977) examined the productivity of glucose isomerase under growth-limiting conditions using O_2 , C, N, P, and Mg as growth-limiting nutrients. Studies were carried out in a 1-liter fermenter with a working volume of 300–350 ml. The impeller speed was varied from 250 to 500 rpm and pH maintained at $7.0 \pm 0.2^\circ C$. The dilution rate was maintained at approximately 0.1 when examining each nutrient limitation. The dissolved oxygen tension was maintained at zero when O_2 was

growth limiting and in other studies at a value higher than 20 mm Hg. The medium used for the oxygen limitation studies comprised $(\text{NH}_4)_2\text{SO}_4$, 2.5 g/liter; K_2HPO_4 , 1.0 g/liter; NaH_2PO_4 ($2\text{H}_2\text{O}$), 1.0 g/liter; NaCl , 0.25 g/liter; MgSO_4 ($7\text{H}_2\text{O}$), 0.1 g/liter; FeCl_3 ($6\text{H}_2\text{O}$), 0.02 g/liter; MnSO_4 (H_2O), 0.02 g/liter; ZnSO_4 , 0.0025 g/liter; CaCl_2 , 0.0075 g/liter; thiamine HCl, 0.001 g/liter; glucose, 8 g/liter; EDTA, 0.083 g/liter; pluronic H-61, 0.083 g/liter. In N_2 , P, and Mg limitation studies the medium was modified accordingly, i.e., $(\text{NH}_4)_2\text{SO}_4$, 0.4 g/liter; K_2HPO_4 , 0.021 g/liter; MgSO_4 ($7\text{H}_2\text{O}$), 0.04 g/liter. The results (Table VI) indicated maximum enzyme yield and productivity when O_2 was limiting.

A summary of medium constituents and growth conditions for the production of glucose isomerase from various strains of *Bacillus* are summarized in Table VII. There is little information available on the fermentation parameters used for large-scale production. Since the enzyme is intracellular, it can be recovered either following lysis of the organism or by harvesting the cells with the enzyme retained within the cells. Originally, the enzyme was utilized in solution but more recently the use of immobilized enzyme preparation or immobilized cells has permitted much shorter processing times and considerably improved the economics of the process. Much of the current production of high fructose/glucose syrups is based on the use of Novo's *B. coagulans* whole-cell preparation.

When the cells are recovered with the intracellular enzyme, considerable loss of enzyme is observed following autolysis of the cells. To avoid this loss of enzyme, and to extend the use of cells in fixed-bed reactors, the enzyme is bound to the intracellular matrix by heat fixation or chemical fixation. Following fermentation and immobilization the cells may be separated from the fermentation broth by centrifugation (Antrim *et al.*, 1979). The Novo process for the immobilization of the enzyme involves crosslinking the harvested cells with glutaraldehyde, breaking them into smaller particles, and drying to improve the mechanical strength of the pellets. Alternative procedures include freezing the crosslinked precipitate prior to drying, allowing the centrifuged cells to autolyse before crosslinking, and then flocculating and drying or disrupting the cells and then flocculating and crosslinking the homogenate. The immobilized enzyme is then used in column reactors at

Table VI. Effect of Nutrient Limitation on the Yield of Glucose Isomerase and Productivity from *B. coagulans*

Dilution rate (D) (hr)	Limiting factor	Yield of cell mass mg dry weight bacteria/mg glucose	Enzyme yield glucose isomerase units/mg dry weight bacteria	Productivity glucose isomerase units/mg glucose/hr
0.12	O_2	0.17	0.98	2.00×10^{-2}
0.12	C	0.29	0.38	1.16×10^{-2}
0.13	N	0.09	0.06	0.07×10^{-2}
0.11	P	0.09	0.17	0.17×10^{-2}
0.10	Mg	0.175	0.11	0.19×10^{-2}

Table VII. Medium Constituents and Growth Conditions for the Production of Glucose Isomerase from *Bacillus* Species

Species	C source	N source	Minerals	pH	Temp	Time	Yield (U/liter of culture)	References
<i>B. coagulans</i> HN-68	Xylose	Yeast extract + NH ₄ Cl	MgSO ₄ (7H ₂ O) MnSO ₄ (4H ₂ O)	—	40	14–16	—	Yoshimura <i>et al.</i> , 1966
<i>B. coagulans</i> NRRL-5650	Glucose	C.S.L. NH ₄ SO ₄	CaCO ₃ MgSO ₄ (7H ₂ O) MnSO ₄ (4H ₂ O) K ₂ HPO ₄	6.8	50	13	8800	Diers, 1977
<i>B. coagulans</i> NRRL-5649-66	Xylose	NH ₄ SO ₄	—	—	60	—	—	Outtrup, 1976
<i>B. steurothermophilus</i>	Xylose	Peptone C.S.L. Yeast extract	NaCl MgSO ₄ (7H ₂ O) CoCl (6H ₂ O)	—	55	—	3890–12,900	Suckane <i>et al.</i> , 1974
<i>Bacillus</i> sp. NRRL B-5350 5351	Xylose Glucose	Meat extract Peptone Casein	—	—	38	17	—	Aunstrup, 1979

about 60°C to produce syrups containing approximately 42–43% (w/w) fructose (Zittan, 1975; Amotz *et al.*, 1976; Hemmingsen, 1979).

3.2. Glycerokinase

Glycerokinase is widely used in clinical chemistry for the determination of serum triglycerides following hydrolysis by lipase to fatty acids and glycerol. The enzyme is produced commercially from a number of mesophilic organisms, such as *E. coli* and *Candida mycoderma*, but such enzymes have proved to be insufficiently stable in storage or under the reaction conditions of the assay. For these reasons a search for a thermophilic source of the enzyme was carried out.

3.2.1. Strain Isolation and Development

The enzyme was found to be produced by *B. stearothermophilus*, "*B. caldotenax*," "*B. caldolyticus*," and "*B. caldovelox*" following growth on a glycerol-containing medium (Atkinson *et al.*, 1975; Comer *et al.*, 1979). Of these four organisms, the wild-type *B. stearothermophilus* NCIB 8924 produced the most glycerokinase and was selected for further study as a source of the enzyme.

The growth medium contained tryptone, 20 g/liter; yeast extract, 10 g/liter; FeCl_3 ($6\text{H}_2\text{O}$), 7 mg/liter; MnCl_2 ($4\text{H}_2\text{O}$), 15 mg/liter; CaSO_4 ($2\text{H}_2\text{O}$), 1.3 g/liter; MgSO_4 ($7\text{H}_2\text{O}$), 0.27 g/liter; citric acid nonohydrate, 0.315 g/liter; Na_2HPO_4 ($2\text{H}_2\text{O}$), 3.2 g/liter. The organism was cycled through liquid culture in a medium containing 40 g/liter sucrose or 40 g/liter glycerol, and then in a medium containing both sucrose and glycerol. Dilution plates using tryptone soya broth agar were prepared from this culture and potential constitutive mutants detected by spraying the plate with a solution containing 50 mg/ml glycerol and 150 mg/ml chloramphenicol. After a further 1-hr incubation at 60°C the plates were sprayed with a solution of 10 g/liter triphenyltetrazolium chloride in 1.0 M phosphate buffer (pH 7.5). Colonies which were constitutive for glycerokinase produced a deep pink color. The colonies showing the deepest pink color were selected for further study and two mutants with enhanced levels of glycerokinase were deposited as *B. stearothermophilus* NCIB 11270 and 11271 (Atkinson and Comer, 1980). The wild-type organism produced about 2 mg enzyme per gram dry weight cells, while the mutants produced between 8 and 12 mg enzyme per gram dry weight (Comer *et al.*, 1979).

3.2.2. Fermentation and Production

Production cultures were grown in a medium similar to that described above, containing tryptone, 2 g/liter; yeast extract, 50 g/liter; FeCl_3 ($6\text{H}_2\text{O}$), 14 mg/liter; MnCl_2 ($4\text{H}_2\text{O}$), 30 mg/liter; MgSO_4 ($7\text{H}_2\text{O}$), 0.54 g/liter; citric acid monohydrate, 0.63 g/liter; Na_2HPO_4 ($2\text{H}_2\text{O}$), 4.9 g/liter (Scawen *et al.*, 1983). Maximum yield of enzyme was obtained by giving a glycerol feed after the dissolved oxygen level of the culture had dropped to about half its initial value. The glycerol concentration in the culture was maintained at about 0.2–0.3 g/liter (Fig. 4). A typical 400-liter

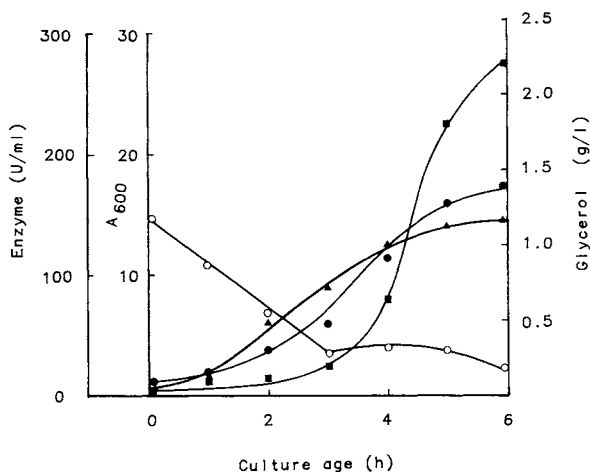


Figure 4. Production of glycerokinase and glucokinase by *B. stearothermophilus* grown in 400-liter culture. (■) Glycerokinase activity; (▲) glucokinase activity; (○) glycerol; (●) A₆₀₀.

culture gave about 10 kg wet bacterial cell paste, containing about 25 g glycerokinase.

3.2.3. Enzyme Recovery and Purification

A typical enzyme purification is based on the bacteria obtained from 800 liters culture, about 20 kg wet weight. The cells were broken using a Manton Gaulin homogenizer and cell debris was removed by centrifugation in a Sharples AS 26 centrifuge. Further cell debris and unwanted proteins were removed by precipitation at pH 5.1. The first procedure described for the purification of the enzymes, based on 1 kg cell paste, utilized batch adsorption and elution from DEAE cellulose at pH 8, precipitation by $(\text{NH}_4)_2\text{SO}_4$, followed by dissolution and desalting of the precipitate. The resulting solution was then further purified by ion exchange chromatography on DEAE Sephadex followed by gel filtration chromatography on Ultrogel AcA 34 (Comer *et al.*, 1979). This procedure, while acceptable for small-scale use, could not be readily adapted to a larger scale. For this reason, an alternative process was developed which used dye affinity chromatography (Table VIII). The supernatant from the pH 5.1 precipitation was adjusted to pH 8.0 and applied directly to a column of DEAE Sephadex, using 2 liters gel for every kilogram of cell paste. The column was eluted with a 5-column-volume gradient of 0.1–0.4 M potassium phosphate buffer at pH 8.0. The enzyme pool was concentrated and desalted by ultrafiltration and applied to a column of Procion blue MX-3G–Sephacrose. The column was washed free of unbound proteins and the glycerokinase eluted by 5 mM ATP. This process gave homogeneous enzyme with a final recovery of about 40% when starting from 20 kg cell paste (Scawen *et al.*, 1983; Hammond *et al.*, 1986).

The enzyme for *B. stearothermophilus* was found to be much more stable than

Table VIII. Purification of Glycerokinase from *B. stearothermophilus*^a

Step	Total protein (g)	Total enzyme (U × 10 ⁻⁶)	Specific activity (U/mg protein)
Homogenate	2540	2.97	1.2
pH 5.1 supernatant	437	2.80	6.4
DEAE Sephadex	65	2.21	34
Procion Blue MX-3G-Sepharose	11	1.34	120

^aData from Scawen *et al.* (1983).

either of the mesophilic enzymes, with a half-life of 5 hr at 70°C compared with less than 5 min for the *E. coli* enzyme and less than 1 min for the *C. mycoderma* enzyme (Comer *et al.*, 1979). In addition, the thermophilic enzyme was found to be very much more stable under the conditions used for the determination of serum triglycerides, where surfactants are employed as activators of the lipase used to hydrolyze the triglycerides (Klose *et al.*, 1981).

3.3. Glucokinase

Hexokinase and glucokinase in conjunction with glucose-6-phosphate dehydrogenase are widely employed for the estimation of glucose. During the purification of glycerokinase from *B. stearothermophilus* the presence of a glucokinase was observed which coeluted from the Procion blue MX-3G-Sepharose column used during the purification of glycerokinase (Scawen and Atkinson, unpublished observations). The presence of this enzyme interfered in glycerol assays and had to be removed (see Section 3.3.2).

3.3.1. Strain Isolation and Development

The strain of *B. stearothermophilus* used (NCIB 11270) was the same as that employed in the production of glycerokinase. Experiments showed that when cultured on glycerol-containing medium this strain gave some sixfold more glucokinase than the wild-type strain cultured on a glucose-containing medium (Goward, 1987), as shown in Table IX. The base complex medium was the same as that

Table IX. Effect of Carbon Source and Strain on the Production of Glucokinase by *B. stearothermophilus*

	Enzyme unit/g dry weight	
Carbon source	NCIB 8928	NCIB 11270
Glycerol	820	940
Glucose	160	390

described for the production of glycerokinase (Section 3.2.). A 400-liter culture produced about 10 kg bacterial cell paste containing about 11 g glucokinase and about 25 g glycerokinase.

3.3.2. Enzyme Recovery and Purification

Because the strain of *B. stearothermophilus* used produced both glycerokinase and glucokinase, and because contaminating glucokinase had to be removed from glycerokinase, a purification protocol suitable for the purification of both enzymes was derived.

The initial steps up to the anion exchange chromatography were the same as those described for the production of glycerokinase (Scawen *et al.*, 1983), although DEAE Sepharose was employed instead of DEAE Sephadex because of the improved flow rate which could be obtained.

Immobilized dyes offer a potentially very high resolution and can be used in a tandem fashion to separate and purify enzymes (see, for example, Clonis *et al.*, 1987). In order to separate glucokinase from glycerokinase, a total of 71 different Procion dye Sepharose conjugates were screened for their ability to bind one enzyme but not the other. One dye, Procion brown H-3R was found which bound glucokinase at about 1 mg enzyme/ml settled gel, but which did not bind glycerokinase (Goward *et al.*, 1986).

The desalted eluate from the DEAE-Sepharose chromatography was applied to a column of Procion brown H-3R-Sepharose. In a tandem purification the outlet from this column was connected directly to the inlet of a Procion blue MX-3G-Sepharose column to bind glycerokinase. The column was washed to remove unbound proteins and the glucokinase eluted with 5 mM ATP. The pooled eluate from this column was further purified by gel filtration chromatography on Ultrogel AcA 34 to give homogeneous enzyme with a final recovery of 38%, as shown in Table X (Goward *et al.*, 1986).

3.4. Leucine Dehydrogenase

Leucine dehydrogenase is used as a reagent enzyme in the estimation of leucine aminopeptidase activity in serum. Leucine aminopeptidase levels are closely associated with pancreatic cancer and are used in the diagnosis and monitoring of

Table X. Purification of Glucokinase from *B. stearothermophilus*^a

Step	Total protein (g)	Total enzyme (U × 10 ⁻⁶)	Specific activity (U/mg protein)
Homogenate	1900	3.61	1.9
pH 5.1 supernatant	640	2.89	4.5
DEAE Sepharose	79	1.88	24
Procion brown H-3R-Sepharose	7.4	1.70	230
Ultrogel AcA 34	4.1	1.37	330

^aData from Goward *et al.* (1986).

this disease. Other bacterial sources of leucine dehydrogenase are known, but they all suffer from poor stability, particularly in storage.

3.4.1. Fermentation and Production

Bacillus stearothermophilus strain ATCC 12980 is grown aerobically at 55°C in a medium containing polypeptone, 12 g/liter; yeast extract, 2.5 g/liter; meat extract, 2 g/liter; glycerol, 2 g/liter; NaCl, 5 g/liter; K_2HPO_4 , 2 g/liter; KH_2PO_4 , 2 g/liter; $MgSO_4 (7H_2O)$, 0.1 g/liter; biotin 4 g/liter.

3.4.2. Enzyme Recovery and Purification

The harvested cells are broken by homogenization and cell debris is removed by centrifugation. Further cell debris and unwanted protein are removed by precipitation with polyethylene glycol. The supernatant is further purified by ion exchange chromatography on DEAE cellulose. Precipitation is accomplished with $(NH_4)_2SO_4$ and gel filtration chromatography on Sephadex G-150 (Oshima and Soda, 1985).

3.5. The Multienzyme Preparation

With the increasing demand for large quantities of many different and stable enzymes for chemical and physical research studies, in particular X-ray crystallography, it became apparent that purifying only one enzyme from a large-scale cell homogenate was uneconomic. For this reason an extraction process was developed which could be used to separate and purify to homogeneity four enzymes, and to partially purify an additional 11 enzymes, from *B. stearothermophilus*. Because most of these enzymes were required for crystallography, it was necessary that the procedures be used on a large scale capable of processing 40–70 kg of bacterial cell paste. Using this large amount of raw material also offered the possibility of isolating significant quantities of enzymes which were present only at low levels in the cell (Atkinson *et al.*, 1979).

Bacillus stearothermophilus was grown at a temperature of 58°C in a medium containing sucrose, 40 g/liter; yeast extract, 50 g/liter; KH_2PO_4 , 10 g/liter; $MgSO_4 (7H_2O)$, 0.54 g/liter; $MnCl_2$, 0.03 g/liter; $FeCl_3 (6H_2O)$, 0.014 g/liter; citric acid monohydrate, 0.64 g/liter, adjusted to pH 7.0 with NaOH. The culture was aerated at 1 vvm at an overpressure of 1 bar and an impeller speed of 200 rpm. Under these conditions a 3000-liter fermentation yielded 35–40 kg of wet cell paste.

The cell paste from two such cultures was broken using a Manton Gaulin homogenizer and the homogenate clarified using a Westphalia disk bowl centrifuge. The clarified extract was purified by a combination of batch ion exchange adsorption to 70 kg DEAE cellulose followed by ion exchange chromatography on an 80-liter column of DEAE Sephadex. The major enzyme pools from this column were then further fractionated by hydroxylapatite chromatography, ion exchange chromatography, or gel filtration chromatography.

Using this process four enzymes—superoxide dismutase, rhodanese, tyrosyl-tRNA synthetase and tryptophanyl-tRNA synthetase—were purified to homogene-

Table XI. Purification of Superoxide Dismutase, Rhodanese, Tyrosyl-tRNA Synthetase and Tryptophanyl-tRNA Synthetase from 70 kg of *B. stearrowthermophilus*^a

Fraction	Total protein (g)	Total units ($\times 10^{-6}$)	Specific activity (U/mg)
Superoxide dismutase			
Cell extract	5600	43	7.7
DE-23 eluate	1850	38	21
DEAE Sephadex	37	29	780
Sephadex G-75	11.2	25	2200
Hydroxylapatite	6.5	19	2900
Rhodanese			
Cell extract	5600	0.69	0.12
DE-23 eluate	1850	0.41	0.22
DEAE Sephadex	242	0.21	0.87
Hydroxylapatite	31	0.092	3.0
Sephadex G-75	4.9	0.059	12.0
pH 5 Supernatant solution	3.0	0.054	18.0
DEAE Sephadex	0.95	0.036	38.0
Sephadex G-75	0.23	0.025	108
Tyrosyl-tRNA synthetase			
Cell extract	5600	0.90	0.16
DE-23 eluate	1850	0.76	0.41
DEAE Sephadex	242	0.62	2.6
Hydroxylapatite	24	0.48	20
DEAE Sephadex	3	0.33	110
Ultrogel	0.7	0.19	272
Tryptophanyl-tRNA synthetase			
Cell extract	5600	0.8	0.14
DE-23 eluate	1850	0.7	0.38
DEAE Sephadex	60	0.46	7.6
Hydroxylapatite	3.1	0.44	141
Aminohexyl-Sepharose	0.4	0.42	1045

^aData from Atkinson *et al.* (1979).

ity (Table XI). Another 11 enzymes, elongation factor Tu, triose phosphate isomerase, alcohol dehydrogenase, phosphogluconate dehydrogenase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, NADH dehydrogenase, and leucyl-, valyl-, and methionyl-tRNA synthetases were all isolated in a form suitable for further purification.

4. SPORULATION PRODUCTS

The genus *Bacillus* is an extremely heterogeneous group of aerobes representatives of which are characterized by their ability to form endospores. Endospores generally do not exist during vegetative growth in complex media. At low growth

rates in synthetic media, however, part of the population may exist as spores. Studies of *B. megaterium* growing in chemostats under glucose limitation indicate that sporulation is induced at a dilution rate of less than 0.5. In normal batch fermentations, most *Bacillus* species form spores as the population enters stationary phase. The maximum rate of synthesis of many extracellular enzymes occurs during the late exponential or early stationary phase growth, as the cells become committed to sporulation (see Chapter 8). The possible relationship between extracellular enzyme synthesis and sporulation has been widely studied. The evidence suggests that extracellular enzymes are not involved in sporulation. Mutants unable to produce α -amylase grow and sporulate normally (Schaeffer, 1969) and mutants of *B. subtilis*, *B. megaterium*, and *B. cereus* which are unable to produce metalloproteases have been shown to grow and sporulate as normal (Millet and Aubert, 1969; Aronson *et al.*, 1971; Hageman and Carlton, 1973). These catabolic enzymes are secreted at the end of exponential growth in response to changing environmental conditions. The synthesis of some antibiotics has also been closely associated with sporulation. Antibiotic-deficient mutants have been isolated which sporulate normally, appearing to exclude any essential role for antibiotics during sporulation. The δ -endotoxin of *B. thuringiensis* is also closely associated with sporulation. Vegetative cells growing on rich media produce neither spores nor δ -endotoxin; however, depletion of the carbon and nitrogen sources from the medium leads to the onset of sporulation followed by the production of the crystal protein (Table XII). Mutants unable to produce the δ -endotoxin, however, still sporulate normally. In addition to the insecticidal δ -endotoxins, spores have a number of commercial applications. Due to their high thermal stability they are used as biological monitors of heat sterilization processes and they have also found application as airborne and waterborne tracers.

4.1. Biotrace

Biotrace is a sensitive biological tracer which can be used to study a variety of water movements and to track discharges into the sea and inland water courses. It is composed of microbial spores of "*Bacillus globigii*" which do not occur in the natural environment, are extremely resistant to environmental conditions, and are non-pathogenic. Biotrace may be used for tracking water movements over long periods and distances. A known amount of material is introduced into the system under investigation and representative samples removed and analyzed to determine the levels present. The spores form bright orange colonies on nutrient agar enabling them to be readily distinguished from other spore-forming organisms which may be present as part of the natural background population. The only alternative trace system known consists of highly specific bacteriophage, which is in the main inherently less stable over prolonged time.

4.1.1. Seed Culture Preparation

Seed inocula are prepared from freeze-dried spore suspensions which are inoculated to each of 10 tryptone soya agar slopes (Oxoid CM131). Following 48 hr incubation at 37°C the slopes are washed with sterile distilled water and the suspen-

Table XII. Media for the Production of δ -Endotoxin from *B. thuringiensis*

Component	Amount (%)	References
Soya flour	2-6	Holmberg <i>et al.</i> , 1980
Molasses	0-4	
KH ₂ PO ₄	0.5	
K ₂ HPO ₄	0.5	
MgSO ₄ (7H ₂ O)	0.005	
MnSO ₄ (4H ₂ O)	0.003	
FeSO ₄ (7H ₂ O)	0.001	
CaCl ₂	0.005	
(Na(NH ₄)PO ₄ (4H ₂ O)	0.15	
Fishmeal	2	Pendleton, 1969
Starch	1	
CaCO ₃	0.1	
Niacin	0.001	Megna, 1963
Beet molasses	1.86 1.0 ^a	
Corn steep solids	1.7 0.85 ^a	
Cottonseed flour	1.4 —	
CaCO ₃	0.1 0.1 ^a	Drake and Smythe, 1963
Cornstarch	6.8	
Corn steep liquor	4.7	
Casein	1.95	
Sucrose	0.64	
Yeast extract	0.6	
Phosphate buffer	0.6	

^aMedia for seed stages.

sion is transferred to a Roux bottle containing 150 ml of tryptone soya agar. After 72 hr incubation the lawn of cells is washed from the agar surface using 50 ml of sterile distilled water (SDW) and glass beads. The washings are bulked to give a suspension of some 40 ml which is heated at 60°C for 30 min to kill vegetative cells. The spore inocula are stored at 4°C until required. The spore suspensions retain high viability, even after several years of storage.

4.1.2. Fermentation

Production batches prepared in 400-liter fermenters are seeded using a 4-liter spore suspension prepared in a 25-liter New Brunswick fermenter. The seed fermenter is inoculated with approximately 1.2×10^{10} spores/ml and a scaled-down process similar to the production fermentation used to provide five to six seed spore stocks for the production fermentation.

The basic medium comprises:

Yeatex (light grade)	50 g/liter
Yeastamine paste	6.25 g/liter
Mazu 8005 antifoam and various mineral nutrients	0.075 ml/liter

Two feeds are made during the fermentation:

1. Mannitol
2. Dextrose monohydrate supplemented with added mineral salts and $\text{CaCl}_2 \cdot 6 \text{H}_2\text{O}$.

The temperature is maintained at 34°C and the pH at 6.2, controlled with 6 N NaOH. Aeration is maintained at 0.75 vvm. Spores become apparent in the fermentation after approximately 16 hr and sporulation is complete after 24–28 hr. After maximum sporulation the culture is heated to 60°C to kill vegetative cells. The spores are harvested using a Westphalia centrifuge. The spores are then resuspended in the culture medium to give a concentration of 1×10^{11} spores/ml.

4.2. Insecticidal Endotoxin

Bacillus thuringiensis was first discovered by the Japanese bacteriologist Ishiwata in 1902, who isolated the organism from diseased silkworms and demonstrated its ability to cause infection. The type strain *B. thuringiensis* var. *thuringiensis* was isolated in Germany by Berliner in 1911 from the Mediterranean flour moth (*Anogosta kuehniella*). It is closely related to *B. cereus* which differs mainly in its ability to synthesize a parasporal protein crystal (α -endotoxin) which is toxic to a variety of insects. The host range of different *B. thuringiensis* preparations are relatively specific, being related only to certain taxonomic groups of insects. *Bacillus thuringiensis* var. *thuringiensis* and var. *kurstaki* are only effective against larvae of Lepidoptera (moths and butterflies) while *B. thuringiensis* var. *israelensis* is effective against larvae of Diptera (flies and mosquitos). Herrnstadt (1986) recently described a strain designated *B. thuringiensis* var. *san diego* which was active against Coleoptera (beetles including boll weevil and the Colorado potato beetle) but did not affect Diptera or Lepidoptera. The ingestion of crystals and in some cases the spores is a prerequisite for pathogenic activity. The crystals become dissolved in the alkaline gut juice of susceptible insect larvae and the proteinaceous toxin is hydrolyzed. The subunits attack the lining of the midgut causing paralysis via leakage of the alkaline gut contents into the hemocele. Lesions may be severe enough to kill the larvae or promote conditions favoring the growth and proliferation of the bacterium resulting in septicemia. The crystal protein from *B. thuringiensis* var. *thuringiensis* has a molecular mass of 130 kDa and is usually cleaved by the larval gut proteases into smaller peptides of 40–60 kDa of which at least one is toxic. Crystals of *B. thuringiensis* var. *israelensis* (25 kDa) are toxic for mosquito larvae without undergoing proteolysis. This mode of action is generally applicable to all *B. thuringiensis*-sensitive insects, although some require infection with spores and crystals when death is the result of septicemia. Crystal synthesis and spore production proceed simultaneously and the two processes appear to be closely linked. Asporogenous mutants generally fail to produce crystals, although mutants producing normal

crystals but failing to complete a sporulation cycle have been isolated (Norris, 1970; Nickerson, 1980). With the majority of strains the spores and crystals separate completely from one another when the remains of the vegetative cell wall are lysed. Bechtel and Bulla (1976) demonstrated from electron microscopy studies that the crystal develops without any visible association with the forespore septum, exosporium, or mesosomes.

4.2.1. Strain Isolation and Development

The principal source of new strains of *B. thuringiensis* is diseased or dead insects; non-spore-formers may be eliminated by heating to 65°C for 15 min. Other spore formers can be reduced by the inclusion of selective antibiotics, including polymyxin B (50 µg/ml), into the nutrient medium. Strains may be cultivated on nutrient agar or tryptone soya agar. Stock cultures are generally maintained as spore suspensions or dried spores which remain viable for several years. The development of new strains requires the use of bioassays for screening activity against a range of different insects. Recent developments in molecular biology have led to the ability to clone the δ -endotoxin gene into other strains of *Bacillus* and into *E. coli*.

Gonzalez *et al.* (1981) implicated specific plasmids in the production of δ -endotoxin by a number of *B. thuringiensis* strains by showing the simultaneous transfer of plasmids and δ -endotoxin from producer strains to strains normally unable to produce crystals. The k-73-type insecticidal protein gene from a 75-kilobase plasmid from *B. thuringiensis* var. *kurstaki* was cloned into *E. coli* pBR322. Subsequent bioassays of the toxin produced by *E. coli* indicated specific activities equivalent to those produced by the parent *B. thuringiensis* strain (McLinden *et al.*, 1985). Similarly, Herrnsstadt *et al.* (1986) cloned a 5.8 kilobase fragment from *B. thuringiensis* var. *san diego* into *E. coli*, again using pBR322 as cloning vector. Purified toxin from the recombinant organism was toxic to the larvae for elm leaf beetle. The technology is therefore now available to carry out production of δ -endotoxin using recombinant microorganisms, offering the possibilities of both enhancing production yields and developing modified toxins with more specific or general insecticidal activity.

4.2.2. Seed Culture Production

Seed cultures for primary stage fermentations are prepared from spore suspensions or deep-frozen spore suspensions stored at -20°C in 10–20% glycerol. Before use, stock cultures should be examined for evidence of crystal formation and must be phage-free. Erlenmeyer flasks containing nutrient broth are inoculated with spores and incubated at 30°C on an orbital incubator shaker at 150–200 rpm for 6–8 hr. Krieg and Miltenburger (1984) recommend a seed medium containing starch, 0.5%; glucose, 0.5%; yeast extract, 0.2%; tryptone, 1.0%, K₂HPO₄, 0.001%; KH₂PO₄, 0.001%. Pflug *et al.* (1981) reported studies of six different culture media for the production of mosquito toxin production using shake-flask cultures of *B. thuringiensis* var. *israelensis*. Buffered medium gave no improvement over unbuffered medium ranging in pH from 5.7 to 8.1 at the end of the culture. A 2% seed inoculum is used to inoculate the primary seed fermenter.

4.2.3. Endotoxin Production

Most production media now utilize complex natural products as sources of carbon, nitrogen, and minerals. Nitrogen sources include fishmeals, cottonseed flour, soya flour, corn steep liquor, autolyzed yeast, and casein. Carbohydrate sources include corn products, starch, dextrose, and beet molasses.

Holmberg *et al.* (1980) described a process scaled up to 1000 liters for the cultivation of *B. thuringiensis* (ATCC 10792). The medium was composed of soya flour, 2–6%; molasses, 0–4%; KH_2PO_4 , 0.5%; K_2HPO_4 , 0.5%; MgSO_4 , 0.5%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0005%; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.003%; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001%; CaCl_2 , 0.005%; $\text{Na}(\text{NH}_4)\text{PO}_4 \cdot 4\text{H}_2\text{O}$, 0.15%. Toxin production was not significantly affected when the pH was varied from 6.5 to 8.0 or aeration varied from 0.1 to 1 vvm. Pendleton (1969) described the production of *B. thuringiensis* in a medium based on fishmeal, 2%; starch, 1%; CaCO_3 , 0.1%; niacin, 0.001%, and a salt mixture. Cultures were scaled up to 12,000 gal; high aeration was considered essential to achieve a high sporulation rate. Growth was carried out at 30°C. Krieg and Miltenburger (1984) described a process which was scaled through 15-, 300-, and 3000-liter fermenters to a 30,000-liter production vessel. Transfer from the seed vessels was carried out at the end of the logarithmic phase of growth (6–9 hr). The yield was dependent on the specific strain and medium used. Megna (1963) recommended a medium containing beet molasses, 1%; corn steep solids, 0.85%; and CaCO_3 , 0.1% for the seed stages and a more concentrated medium composed of beet molasses, 1.86%; corn steep solids, 1.7%; cotton seed flour, 1.4%, and CaCO_3 , 0.1% for the production fermenter. The spore yields on this medium were in the region of 5×10^9 spores/ml. Drake and Smythe (1963) described a medium comprising cornstarch, 6.8%; corn steep liquor, 4.7%; casein, 1.95%; sucrose, 0.64%; yeast extract, 0.6%; and a phosphate buffer, 0.6%. To stimulate growth and to maximize sporulation and crystal formation in the stationary phase, aeration of the culture is essential and should be maintained at 0.3 vvm; dissolved oxygen should not fall below 20%. Fermentation generally takes from 36 to 48 hr to maximize crystal formation. Monitoring of the process is carried out by total counts of the vegetative cells and spores. Spore/crystal ratios are monitored during the final stages of the fermentation and with *B. thuringiensis* var. *kurstaki* give a value of 0.5 at the end of the process. Typical growth kinetics for *B. thuringiensis* var. *kurstaki* are presented in Fig. 5. Optimum toxin production is based on the crystal protein concentration or the number of crystals observed microscopically in a representative number of spores. More direct assays have been based primarily on bioassays performed by adding dilutions of the endotoxin preparation to an artificial diet and feeding susceptible larvae (Dulmage *et al.*, 1971; Moraes and Chaib, 1978). Bioassays generally take from 4 to 7 days and require considerable labor in maintaining, rearing, and testing suitable insect larvae. Immuno-electrophoretic assays (Winkler *et al.*, 1971) reduced the assay period to 48 hr but the time to carry out the assay was still longer than the duration of the fermentation. Andrews *et al.* (1980) developed a rocket immuno-electrophoresis assay which gave results in 4 hr. The assay was considered to be essentially qualitative and required a considerable amount of antigen (10 $\mu\text{g}/\text{ml}$). More recently, ELISA techniques were devised which show very close correlation with results from bioassays and give reliable results within 4 hr (Smith and Ulrich, 1983).

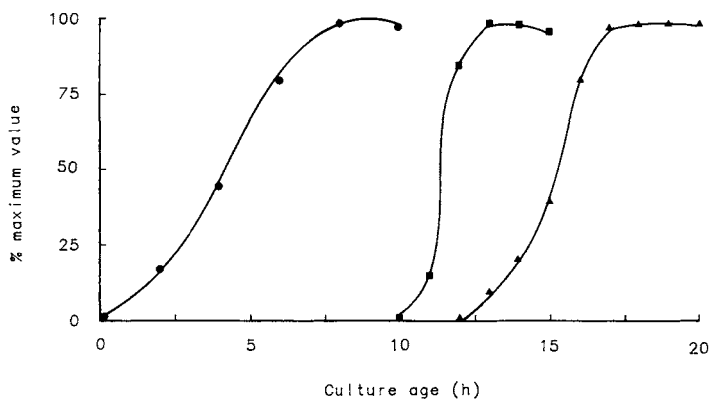


Figure 5. Growth, crystal formation, and sporulation in *B. thuringiensis*. (●) Cell growth; (■) crystal formation; (▲) spores.

4.2.4. Harvesting and Formulation

At the end of crystal formation in the fermenter the spores and crystals are washed and concentrated by centrifugation. Zamola *et al.* (1981) discussed the effects on the insecticidal activity of continuous centrifugation for the separation of crystals and spores from the medium. Studies using a solid bowl disk centrifuge and an intermittent discharge centrifuge indicated that the insecticidal activity of the cell paste was dependent not only on the course of the fermentation but on the flow rate through the centrifuge. After washing the paste may be filtered to produce a moist filter cake which is then dried in a vacuum oven (Pendleton, 1969) or spray-dried to produce the primary powder. After drying the preparation is formulated to ensure sufficient dispersion to give an acceptable dosage of crystals when applied in the field. It is generally prepared as a wettable powder suspended in water with an inert hydrophilic carrier such as bentonite. Other additives to aid adhesion or dispersion, or to act as attractants, may be added prior to spraying.

5. RECOMBINANT PRODUCTS

The potential of members of the genus *Bacillus* as hosts for the cloning and expression of heterologous genes has been well documented (Young, 1980; Mezes and Lampen, 1985). Their major potential lies in their almost unparalleled ability to translocate and secrete specific protein products into the culture medium where they can accumulate. Thus, much attention has been directed to the development of secretion vectors in which the coding sequences for the secretory signal peptides of α -amylase, β -lactamase (penicillinase), or the alkaline protease (subtilisin) are fused to the coding sequence of the product's structural gene. The inherent danger in this approach, however, particularly from *B. subtilis*, *B. amyloliquefaciens*, and *B. licheniformis*, has been the inevitable presence of extracellular, highly active neutral, and especially alkaline proteases capable of degrading or at least "nicking" the

recombinant product. Recent work has therefore been directed at the development of "protease-deficient" or "protease-minus" strains of the above, and more recently attention has also been given to the naturally occurring, low-protease species *B. brevis* and *B. megaterium* as hosts for recombinant products. In addition, thermophilic species of *Bacillus* such as *B. stearothermophilus* have also been investigated as hosts for thermostable recombinant products.

The development of adequate cloning and expression systems in the 1970s and 1980s are detailed elsewhere in this book (see Chapter 5). More recent work related to the production of recombinant proteins has been directed at understanding the secretory mechanism itself using α -amylase or β -lactamase as model systems, in order to combat the lack of secretion observed with early recombinant products such as the major antigen of foot-and-mouth virus and hepatitis B core antigen (Hardy *et al.*, 1981). The development and potential of secretory vectors and systems using α -amylase particularly and β -lactamase have been well reviewed by Mezes and Lampen (1985).

The successful production of cloned *B. amyloliquefaciens* α -amylase from *B. subtilis* with production levels of five times the donor strain has been demonstrated (Palva, 1982). Similarly elevated expression of the *B. licheniformis* β -lactamase from *B. subtilis* has been previously demonstrated (Gray and Chang, 1981). Production of both of these proteins was reviewed in Section 2.

Although the secretion into the *Bacillus* culture medium of *foreign* gene products correctly processed at the N terminus has proven to be attainable and has indicated that the industrial production of such products might be a realistic aim, relatively little success has been obtained in converting such work into a production system. Thus, the *B. amyloliquefaciens* α -amylase secretion vector gave correctly processed human α_2 -interferon in the culture medium of *B. subtilis* (Palva *et al.*, 1983). Unfortunately, yields of 0.5–1 mg/liter ($1\text{--}2 \times 10^8$ U/liter) culture are lower than those attainable with other systems and even though a protease-deficient *B. subtilis* host was used, the α_2 -interferon product was rapidly degraded as the culture neared stationary phase. Subsequent work with a similar vector construct in continuous culture at a relatively low growth rate ($\mu = 0.14$ hr⁻¹) gave extracellular α_2 -interferon titers of 1.4×10^7 U/liter per hour and substantially avoided the problem of degradation by proteolysis (Meyer and Fiechter, 1985).

A relatively novel approach to producing recombinant proteins from *B. subtilis* was demonstrated by Mosbach *et al.* (1983), who immobilized rat proinsulin producing *B. subtilis* cells in 2% agarose. By using novobiocin to arrest cell division, a system was established which secreted authentic proinsulin at 7–10 μ g/liter for many hours from a genetic construct in *B. subtilis* consisting of the β -lactamase signal sequence fused to the rat preproinsulin gene.

Intracellular production in *B. subtilis* of foreign gene products has also been demonstrated. Thus the *B. sphaericus* gene coding for the synthesis of penicillin V amidase, which catalyzes the hydrolysis of penicillin V to 6-aminopenicillanic and phenoxyacetic acids, has been cloned into *B. subtilis* and from its own promoter produced twice the level in *B. subtilis* of the donor (Olssen *et al.*, 1985).

Perhaps one of the major recent production systems derived in *B. subtilis* has gone largely unreported because of commercial pressures. The *S. aureus* gene for protein A has now been cloned in organisms including *E. coli*, *S. aureus*, and *B. subtilis* (Uhlen *et al.*, 1984; Fahnestock, 1986; Shuttlesworth *et al.*, 1987). Available

data indicate that gram per liter production of protein A, intracellularly with *E. coli* but extracellularly with *S. aureus* and *B. subtilis*, has been achieved. Detailed publication of these systems is eagerly awaited.

Finally, site-directed mutagenesis, most notably of the *Bacillus* alkaline serine protease, subtilisin, is ongoing in programs in both the USA and the UK. Whether new catalytic activities or enhanced stability leading to a new range of proteases possessing modified activities can be engineered into this protein is an area for future study.

6. CONCLUSION

Major production from and in *Bacillus* species of industrial proteins has been established for many years. Early products such as α -amylase and neutral and alkaline proteases, required on the multi-kilogram scale, have been joined by similar products such as *B. thuringiensis* crystal toxin and small quantities of low volume, higher-value products such as diagnostic enzymes from *B. stearothermophilus*. It remains to be seen if the advent of recombinant DNA technology will have a major impact on the production of foreign products in or from the *Bacillus* species.

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Products and Applications

11

FERGUS G. PRIEST

1. INTRODUCTION

Aerobic endospore-forming bacteria of the genus *Bacillus* have had a long, but until relatively recently restricted, involvement in applied microbiology. The solid-state fermentation of soybeans by *Bacillus subtilis* to produce “natto” has been exploited in Japan for over a thousand years (Hara and Veda, 1982) and various *Bacillus* species are involved in the fermentation of cocoa beans, a process that dates back several centuries (Carr, 1983). This long association of bacilli with human foods assures the safety of the majority of these bacteria (*B. anthracis* and food-poisoning strains of *B. cereus* being notable exceptions) and has eased their commercial exploitation. Indeed, *B. subtilis* and *B. licheniformis* are “generally regarded as safe” and qualify for GRAS clearance by the Food and Drug Administration (FDA) in the United States, an important consideration if these bacteria are to be used to produce materials destined for human consumption.

Bacillus species have played an important and expanding role in the development of applied microbiology during this century. Industrial enzymes, initially used as aids to digestion but later for food processing and other applications, were introduced in the early 1900s. *Bacillus amyloliquefaciens*, at that time confused with *B. subtilis*, became the dominant source of α -amylase and today with *B. licheniformis* accounts for much of the total world output of industrial enzymes. Later, developments in biotechnology included the discovery and production of antibiotics in which *Bacillus* was to play a relatively small but important part. Biological control of insect pests and vectors of disease is a relatively recent commercial innovation although the principles have been considered for many years. *Bacillus thuringiensis* and other insect pathogenic *Bacillus* strains are key intermediaries in this fast-developing industry. With such a pedigree, it is not surprising that *B. subtilis* and its relatives are attracting considerable attention in the development of many aspects of biotechnology. This chapter reviews the background to the current uses of *Bacillus* in industrial microbiology and hopefully indicates some of the possibilities for further exploitation of this remarkable genus.

2. ENZYMES

2.1. Distribution and Screening of Extracellular Enzymes within the Genus

The aerobic, endospore-forming bacteria are a rich source of extracellular depolymerases active on carbohydrates, proteins, nucleic acids, and various miscellaneous compounds (Priest, 1977). Since some of these enzymes form the basis of traditional taxonomic tests, their distribution among the species is reasonably well documented. Table I lists some of the more important enzymes but it should be

Table I. Some Important Extracellular Enzymes from *Bacillus*^a

Enzyme	Species	Comments
Starch-hydrolyzing enzymes		
α -Amylase	<i>B. amyloliquefaciens</i>	The tradition "industrial" amylase
	<i>B. licheniformis</i>	Thermostable industrial amylase
	<i>B. acidocaldarius</i>	pH optima of 2–4.5, depending on strain
	" <i>B. alcalophilus</i> sub-species <i>halodurans</i> "	pH optimum about 9.0
β -Amylase	<i>B. cereus</i>	Optimum temperature about 50°C
	<i>B. megaterium</i>	Optimum temperature about 50°C
	<i>B. polymyxa</i>	Optimum temperature about 37°C
" β -Amylase"	<i>B. stearothermophilus</i>	Exo-attacking, producing α -maltose from starch
Isoamylase	<i>B. amyloliquefaciens</i>	Hydrolyzes amylopectin and glycogen but not pullulan
Pullulanase	" <i>B. acidiopullulyticus</i> "	Hydrolyzes the 1,6- α branches in amylopectin
	<i>B. cereus</i>	
	<i>B. polymyxa</i>	
Proteases		
Metalloprotease	<i>B. amyloliquefaciens</i>	The traditional industrial enzyme
	" <i>B. thermoproteolyticus</i> "	Thermostable industrial enzyme
Serine protease	<i>B. licheniformis</i>	Temperature-stable, high pH optimum; used in detergents
	Alkaliphilic bacilli	Improved alkaline stability for inclusion in detergents
Penicillinases		
β -Lactamase	<i>B. cereus</i>	Inactivates penicillins
	<i>B. licheniformis</i>	
Penicillin amidase (acylase)	<i>B. megaterium</i>	Used for synthesis of semisynthetic penicillins
β -Glucanase	<i>B. amyloliquefaciens</i>	Commercial preparations used in brewing and related industries
	<i>B. subtilis</i>	
Glucose isomerase	<i>B. coagulans</i>	Intracellular enzyme used in immobilized form for fructose manufacture from glucose

^aData prepared from Priest (1977), Fogarty (1983), and Kindle (1983).

remembered that in few cases have the enzymes been characterized. Thus starch degradation could refer to an α - or β -amylase or a glucoamylase type of enzyme.

Screening tests for extracellular enzymes often depend on the solubilization of an insoluble substrate such as casein in skimmed milk or chitin. For soluble substrates, the polymer can be precipitated or stained to reveal clearing zones around colonies as in the traditional test for starch hydrolysis using iodine solution or vapor. A popular procedure is to couple the substrate to a dye such as remazol brilliant blue or azure. As the insoluble, dyed substrate is hydrolyzed, low molecular weight products are released and dissolve leaving pale or colorless zones around enzyme-producing colonies. This process generally distinguishes exo-attacking from endo-attacking enzymes since the bound dye molecules inhibit the movement of the exo-attacking enzyme along the polymer chain.

Enzymes that act on low molecular weight substrates can often be detected using assay substrates such as nitrophenyl and methylumbelliferyl derivatives of sugars. These screens are not so straightforward since the substrates and products are readily diffusible in agar media and zones around colonies are indistinct. This can be partially overcome by using substrates in agar overlays, but conditions need to be optimized for individual enzymes. A comprehensive list of screening and detection procedures for extracellular enzymes has been published recently (Priest, 1984).

2.2. *Bacillus* Enzymes and Their Commercial Applications

2.2.1. Proteases

Most strains of *Bacillus* hydrolyze gelatin and/or casein although the enzymes responsible have been purified and characterized in relatively few cases. The most satisfactory classification of proteases is that based on their catalytic properties, which results in four groups: the serine proteases, metalloproteases, cysteine proteases, and aspartic proteases (Hartley, 1960). These can be distinguished by their sensitivity to various inhibitors (North, 1982). Thus the serine proteases are inhibited by phenylmethylsulfonylfluoride (PMSF) and diisopropylfluorophosphate (DIFP), the metalloproteases by chelating agents, the cysteine enzymes by iodoacetamide and heavy metals, and the aspartic proteases by pepstatin. In protease mixtures, these inhibitors give the opportunity of assaying individual classes of enzyme in the absence of purification. This classification of proteases correlates well with their pH optima. Thus the serine proteases have alkaline pH optima, the metalloproteases are optimally active around neutrality, and the cysteine and aspartic enzymes have acidic pH optima. The available evidence suggests that strains of *Bacillus* secrete metallo- and/or serine proteases but the acid proteases, which have considerable commercial potential as substitutes for rennet in cheese manufacture, are largely restricted to the fungi and higher eukaryotes (Ward, 1983).

2.2.1a. Assay of protease activity. There are several common assay procedures for protease based on the release of acid-soluble peptides from casein or hemoglobin measured as absorbance at 280 nm or colorimetrically using the Folin method (Bergmeyer, *et al.*, 1984). These have largely been superseded by methods involving the solubilization of chromogenic substrates such as hide powder azure or azocoll (Ward, 1983). Specific assays for neutral protease include using furylacrylyl-

glycyl-leucine amide or PZ-peptide as substrate (Keay, 1972) and for serine protease succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide is very sensitive (Del Mar *et al.*, 1979).

2.2.1b. Metalloproteases. Extracellular metalloproteases have been isolated and characterized from *B. amyloliquefaciens*, *B. cereus*, *B. megaterium*, *B. polymyxa*, *B. subtilis*, *B. thuringiensis*, and "*B. thermoproteolyticus*" (probably a strain of *B. stearothermophils*) (reviewed by Keay, 1972; Priest, 1977; Ward, 1983). These enzymes have pH optima at or just above neutrality, a molecular mass of 35,000–40,000, and require zinc for activity and calcium for stability. They are endo-acting enzymes that preferentially cleave bonds with the amino group of leucine or phenylalanine. With the exception of the enzyme from "*B. thermoproteolyticus*," these enzymes are temperature-labile; on average, a 50% loss of activity occurs during 15 min incubation at 59°C. Commercially metalloprotease is produced from *B. amyloliquefaciens*, which is grown in conditions that repress α -amylase and serine protease synthesis, and from "*B. thermoproteolyticus*." The enzyme is used in leather bating and for preventing protein hazes forming in beer. It is also useful for reducing the gluten content of flours used in the production of biscuits and cookies (Ward, 1983).

2.2.1c. Serine proteases. Serine proteases are secreted by *B. amyloliquefaciens*, *B. licheniformis*, *B. subtilis*, and *B. pumilus*. These are smaller than the metalloproteases (25,000–30,000 Da), exhibit maximum activity at pH 9.0–11.0, and hydrolyze simple terminal esters. They have no metal ion requirement and resemble the animal enzyme trypsin. These enzymes can be divided into two groups; those from *B. licheniformis* (subtilisin Carlsberg) and *B. pumilus* are less stable below pH 9.0 and have higher esterase activity than those from *B. amyloliquefaciens* (subtilisin Novo; subtilisin BPN) and *B. subtilis* (Keay, 1972). The DNA sequence of the gene from *B. subtilis* is 80% homologous with that from *B. amyloliquefaciens* (Wells *et al.*, 1983) and the translated sequence from *B. subtilis* is 85% homologous with the amino acid sequence of subtilisin BPN (Stahl and Ferrari, 1984).

These enzymes are particularly well suited for inclusion in household washing detergents; their major application. The search for improved temperature and alkali stability encouraged the isolation and examination of alkaliphilic strains of *Bacillus*. Such strains are common and appear to be taxonomically related to *B. lentus* and *B. firmus* (Gordon and Hyde, 1982), although some have been given species status (e.g., *B. alcalophilus*). Serine proteases from these bacteria are active under strongly alkaline conditions (up to about pH 12). In detergents they are often superior to the enzymes from *B. licheniformis* and their high pH stability favors their use for dehairing hides in the leather industry (Aunstrup, 1980).

2.2.2. Starch Hydrolyzing Enzymes

Strains of many *Bacillus* species secrete starch-hydrolyzing enzymes (Table I) and, although few have been characterized, a wide variety of enzymes appears to be represented. Amylases that hydrolyze internal 1,4- α linkages in amylose and amylopectin are termed α -amylases. These enzymes cause a rapid reduction in both the viscosity and iodine-staining capacity of the substrate with little release of reducing power. The final products of starch hydrolysis are malto-oligosaccharides of

two to six units in length depending on the origin and nature of the amylase and a variety of branched α -limit dextrans that contain the 1,6- α linkages from the original polymer (Fogarty, 1983; Priest, 1984).

Exo-attacking amylases that hydrolyze alternate 1,4- α linkages in starch to yield maltose in the β configuration and a β -limit dextrin are termed β -amylase. These enzymes can be distinguished from α -amylase by the rapid release of reducing sugars from starch accompanied by a slow reduction in viscosity or iodine-staining power (Thomas *et al.*, 1980). Glucoamylase (also known as amyloglucosidase) is an exo-acting enzyme that yields β -D-glucose by hydrolysis of the 1,4- α linkages in starch. It also hydrolyzes the 1,6- α linkages of amylopectin albeit rather slowly. It is of fungal origin and is rarely synthesized by bacteria (Fogarty, 1983).

Debranching enzymes specifically hydrolyse the 1,6- α linkages of amylopectin and glycogen. Debranching enzymes from *Bacillus* include pullulanases that hydrolyze the 1,6- α bonds of pullulan and amylopectin but have little activity on glycogen and isoamylase which does not attack pullan but hydrolyzes amylopectin and glycogen.

2.2.2a. Amylase assays. There are almost as many assays for amylases as there are enzymes. Common procedures for α -amylase use the reduction in iodine-staining of starch, which is rapid but not particularly accurate, or the increase in reducing power upon digestion of starch. Substituted starches such as starch azure provide rapid, simple measures of α -amylase activity but cannot be used for exo-attacking enzymes since the dye molecules inhibit the progress of the enzyme along the molecule. These substrates therefore provide the possibility of estimating α -amylase in the presence of β -amylase but there is no equivalent for the assay of β -amylase in the presence of α -amylase. Pullulanase is easily measured as the release of maltotriose (as reducing power) from pullulan (Adams and Priest, 1977) but isoamylase can only be estimated as the *increase* in iodine-staining power upon digestion of glycogen (Urlaub and Wober, 1975).

2.2.2b. α -Amylase. α -Amylase is secreted by *B. alcalophilus*, *B. acidocaldarius*, *B. amyloliquefaciens*, "*B. caldolyticus*," *B. circulans*, *B. coagulans*, *B. licheniformis*, *B. macerans*, *B. megaterium*, *B. stearothermophilus*, *B. subtilis*, and *B. thuringiensis* (reviewed by Priest, 1977; Ingle and Erickson, 1978; Boyer *et al.*, 1979; Kindle, 1983; Fogarty, 1983). These enzymes vary widely in their pH and temperature optima for activity and the size of limit dextrans produced from amylose. Only the enzymes from *B. amyloliquefaciens* and *B. licheniformis* are produced on a large commercial scale. Their main use is for the liquefaction of starch prior to saccharification for the production of sugar syrups (Fogarty, 1983). The α -amylase from *B. amyloliquefaciens* once dominated this market but the exceptional temperature stability of the *B. licheniformis* enzyme makes this the first choice in most instances (Madeson *et al.*, 1973).

2.2.2c. β -Amylase. Maltose syrups have the desirable properties of low viscosity and hygroscopicity as well as good heat stability, and are useful in the confectionary and baking industries. Although β -amylases are secreted by several strains of *Bacillus* including *B. cereus* var. *mycoides*, *B. megaterium*, *B. circulans*, and *B. polymyxa*, these enzymes generally resemble plant β -amylase and are too temperature-labile for industrial usage (reviewed by Fogarty, 1983). More recently, a " β -

amylase" from *B. stearothermophilus* that has an exo mode of attack but yields α -maltose has been described and is sufficiently temperature-stable to permit commercialization (Outtrup and Norman, 1984).

2.2.2d. Debranching enzymes. Many amylolytic strains of *Bacillus* can degrade pullulan (Table I; Morgan *et al.*, 1979), and presumably secrete the debranching enzyme pullulanase. The enzymes from *B. cereus*, *B. macerans*, and *B. polymyxa* have been characterized (Fogarty, 1983) and the pullulanases from "*B. acidopullulyticus*" (Jensen and Norman, 1984) and *Klebsiella pneumoniae* are produced commercially. Isoamylase is synthesized by *B. amyloliquefaciens* (Urlaub and Wober, 1975). Pullulanases are used in the starch industry to assist in the production of sugar syrups (Jensen and Norman, 1984).

2.2.2e. Glucose isomerase. Although not a starch-degrading or extracellular enzyme, glucose isomerase is mentioned here because in its immobilized form it is used by the starch industry in the manufacture of high-fructose corn syrups (HFCS). Fructose is considerably sweeter to taste than sucrose and is used as a sweetener in beverages and foods. Briefly, starch is hydrolyzed to dextrins using bacterial and fungal α -amylases and glucoamylase is used to hydrolyze this material to glucose. The glucose is converted into a high-fructose syrups in a reversible reaction using immobilized D-xylose isomerase from *B. coagulans* or various actinomycetes. This is the first example of industrial utilization of an immobilized enzyme and it has become a large-volume business since the incorporation of HFCS in various carbonated beverages.

2.2.3. Other Extracellular Enzymes

In addition to the amylases and proteases, β -glucanase is prepared commercially from strains of *B. amyloliquefaciens* and *B. circulans* (Priest, 1984). Pectin-degrading enzymes are widely distributed throughout the genus (Table I; Fogarty and Kelly, 1983). Like other bacterial pectinases, these are largely polygalacturonate *trans*-eliminases and the rate and extent of activity of these enzymes decreases with increasing esterification of the substrate. Pectin lyases have more commercial application and are generally produced from fungi (Fogarty and Kelly, 1983).

Thus the genus *Bacillus* is an abundant source of extracellular enzymes, and the recent commercial introduction of pullulanase from "*B. acidopullulyticus*" and the discovery of β -amylase-like enzymes in *B. stearothermophilus* indicate that novel and useful enzymes remain to be discovered.

2.3. Protein Secretion

Protein secretion is usually defined as the export of a protein across the cytoplasmic membrane; the final location of this enzyme will therefore largely depend on the structure of the cell envelope. In the typical gram-positive bacterium such as *Bacillus*, the cell wall may not offer a barrier to permeability (but see Chapter 9, Section 6) and the enzymes generally diffuse into the external medium although some enzymes, e.g., alkaline phosphatase and penicillinase are bound to

the cytoplasmic membrane (see Section 2.3.2). In gram-negative bacteria, the outer membrane prevents diffusion of these exported proteins, which are consequently restricted to the periplasm. However, some enzymes are completely externalized, particularly in pseudomonads and some enterobacteria such as *Serratia* and *Erwinia*.

2.3.1. Signal Hypothesis

The signal hypothesis, originally proposed by Blobel and Sabatini (1971) to explain how proteins are localized within eukaryotic cells, is now fully substantiated, albeit sometimes in modified form, for most examples of protein secretion in eukaryotes and prokaryotes. Briefly, exported proteins are synthesized in precursor form and bear an amino terminal extension of some 15–32 amino acids—the signal peptide. As it emerges from the ribosome, this peptide interacts with the inner side of the cytoplasmic membrane, possibly with the help of membrane-located receptor proteins. In eukaryotic cells a signal recognition particle has been identified that temporarily halts translocation once the signal peptide has emerged and interacts with a “docking” (receptor) protein in the membrane which allows translation to ensue (Meyer *et al.*, 1982). The *sec* mutations of *E. coli* (Silhavy *et al.*, 1983) and the 64 kDa protein from ribosome membrane complexes of *B. subtilis* (Horiuchi *et al.*, 1983) might define similar components in prokaryotes. With the ribosome associated with the membrane, the polypeptide chain traverses the membrane as it is synthesized in a process termed “cotranslational secretion”. There is mounting evidence for a secretory machinery housed in the membrane of *E. coli* that effects this transport (Michaelis and Beckwith, 1982). After synthesis, a specific protease, the signal peptidase, removes the signal peptide thus allowing the enzyme to assume its final configuration on the outside of the membrane. The experimental evidence in support of this process is now overwhelming and has been reviewed extensively (Blobel *et al.*, 1979; Wickner, 1979; Davis and Tai, 1980; Inouye and Halegoua, 1980; Emr and Silhavy, 1982; Michaelis and Beckwith, 1982; Silhavy *et al.*, 1983; Briggs and Gierasch, 1986).

The structures of several signal peptides of secreted enzymes from *Bacillus* species have now been established largely as inferred sequences from DNA nucleotide sequences. The α -amylases of *B. amyloliquefaciens* (Palva *et al.*, 1981; Takinen *et al.*, 1983), *B. subtilis* (Yang *et al.*, 1983; Yamazaki *et al.*, 1983), and *B. licheniformis* (Stephens *et al.*, 1984) have signal peptides of 31, 41, and 29 amino acid residues, respectively, immediately preceding the mature amylase protein. Although there is little direct DNA homology between them, they each have the typical structure comprising of a positively charged NH_2 terminal end of about 10 amino acids followed by a substantial hydrophobic domain of about 20 residues before the coding region of the mature protein. The serine proteases of *B. amyloliquefaciens* (Wells *et al.*, 1983; Vasantha *et al.*, 1984) and *B. subtilis* (Stahl and Ferrari, 1984; Wong *et al.*, 1984) and the metalloproteases of these organisms (Vasantha *et al.*, 1984; Yang *et al.*, 1984) and *B. stearothermophilus* (Fujii *et al.*, 1983) have been cloned and analyzed. Again, the signal peptides which have been inferred from DNA sequences have the typical distribution of polar and hydrophobic amino acid residues found in other systems. In general it seems that gram-positive signal peptides are considerably longer and more positively charged at the NH_2 terminal end than the signal peptides characteristic of *E. coli*.

An interesting feature of both the serine and metalloproteases that has been revealed by fine structure analysis of the genes is the presence of a long reading frame between the signal sequence and the mature protein sequence which codes for about 77 amino acid residues in the case of the serine protease of *B. amyloliquefaciens* to 194 for the metalloprotease of this bacterium. This sequences referred to as the "pro" sequence, may be responsible for reducing the activity of the intracellular enzyme as in the zymogens of eukaryotes or alternatively it might be involved in binding the proenzyme to the membrane during export (Vasantha *et al.*, 1984; Yang *et al.*, 1984).

2.3.2. Membrane-Bound Intermediates

Some enzymes, in particular the alkaline phosphatases of *B. subtilis* and *B. licheniformis* and the penicillinases of *B. licheniformis* and *B. cereus*, are distributed in various locations. Alkaline phosphatase (reviewed by Hulet, 1986) has been identified in fully secreted form (Spencer *et al.*, 1981), a cell-bound form released by lysozyme (Hansa *et al.*, 1981), a salt-extractable membrane form (Schaffel and Hulet, 1978), a detergent-extractable membrane form (Spencer *et al.*, 1981), and an enzymatically inactive form in the cytosol (Hulet, 1984). Similarly, in a mutant of this bacterium constitutive for penicillinase synthesis, about half of the total penicillinase exists on the outer surface of the cytoplasmic membrane as a strongly hydrophobic form and the rest is totally extracellular in a soluble hydrophilic form of two molecular mass classes (Izui *et al.*, 1980; Lampen and Nielsen, 1982). The membrane form is analogous to lipoprotein of *E. coli* since it contains an amino terminal glyceride thioether modification linked through a cysteine residue which results in the hydrophobicity and anchorage to the membrane. Specific proteolytic cleavage removes this anchor sequence releasing the hydrophilic enzyme (Lampen and Nielsen, 1982). It seems that such glyceride-cysteine lipoproteins may constitute a class of membrane enzymes analogous to the periplasmic enzymes of gram-negative bacteria (Nielsen and Lampen, 1982). In addition to the lipophilic segment, penicillinase is synthesized initially with an amino terminal signal peptide of 34 amino acids (Kroyer and Chang, 1981).

2.3.3. Secretion of Foreign Proteins

Two important principles emerge from this brief consideration of protein secretion. First, a signal sequence seems sufficient to at least initiate the process of export for any protein and in many cases results in complete secretion. Second, protein export has been highly conserved throughout evolution to the extent that prokaryotic cells recognize and process eukaryotic signal peptides and vice versa. The possibility arises that potentially any protein can be secreted by gram-positive bacteria such as *Bacillus*, although in *E. coli* these proteins would probably accumulate in the periplasm. This has encouraged the use of *B. subtilis* as a host for cloning genes for industrial purposes (see Chapter 5, Section 8).

Extracellular proteins have several advantages in biotechnology. Ease of recovery and purification and the potential for extremely high yields are important considerations in the cost-effectiveness of all production processes. A major draw-

back in *Bacillus* strains is the prevalence of extracellular proteases that readily degrade foreign proteins, particularly as the culture enters stationary phase. Although originally thought an intractable problem, recent cloning and *in vitro*-derived deletions of the serine (Stahl and Ferrari, 1984) and metal protease (Yang *et al.*, 1984) genes has enabled the construction of virtually protease-free strains. Such strains have been used for the production of cloned human growth hormone and, unlike the parental host strain, no loss nor proteolytic degradation of the product occurred even during stationary phase growth (Ruppen *et al.*, 1986).

2.4. Regulation of Synthesis

The synthesis of extracellular enzymes in *Bacillus* is governed by a complex control system that can operate at at least three levels. First, many enzymes are repressed during exponential growth and synthesized only as the culture enters the stationary phase of growth. This appears to be a separate phenomenon from carbon and nitrogen catabolite repression, which control the synthesis of most if not all extracellular enzymes. Finally, some extracellular enzymes are inducible in a fashion analogous to that of the typical inducible cytoplasmic catabolic enzyme.

2.4.1. Physiological Functions of Extracellular Enzymes

At the end of batch culture growth, strains of *Bacillus* undergo a complex process of differentiation resulting in the endospore (see Chapter 8). Since many, but not all, extracellular enzymes are synthesized just prior to or as the culture sporulates, there has been considerable debate over a functional role for these enzymes in sporulation (Schaeffer, 1969; Priest, 1977; see Chapter 8). The isolation of amylase- and metalloprotease-deficient mutants that sporulated normally divorced these enzymes from sporulation but mutations of the serine protease that were nonpleiotropic and sporulated normally proved extraordinarily difficult to obtain. The recent cloning of this enzyme from *B. subtilis* has enabled *in vitro* mutagenesis and it is now clear that mutants, completely defective for this enzyme, grow and sporulate normally (Stahl and Ferrari, 1984). Thus no extracellular enzymes of *B. subtilis* play any part in sporulation and they can be regarded simply as "scavenger" enzymes that degrade polymeric molecules in the environment that are too large to enter the cell. Nevertheless, it seems that the control of their synthesis is integrated into the control of sporulation (see Chapter 8) with serine protease being the most closely coupled since it is transcribed by σ^{37} -containing RNA polymerase.

2.4.2. Nitrogen and Catabolite Repression

Catabolite repression, the inhibition of synthesis of catabolic enzymes for "poor" carbon sources by rapid growth on a "good" carbon source, usually regulates extracellular enzyme synthesis (reviewed by Priest, 1984). In gram-negative bacteria such as *E. coli*, cyclic AMP and the activator protein CAP account for most aspects of catabolite repression at the molecular level but many gram-positive bacteria, notably *Bacillus*, do not contain cyclic AMP or the enzyme responsible for its synthesis (see Priest, 1977). *Bacillus circulans* is an exception and cyclic AMP has

been implicated as a negative effector and prevents the induction of β -glucanase synthesis by 1,3- β -glucan (Esteban *et al.*, 1984a). However, only one of the four forms of the enzyme was affected and it seems unlikely that this is related to catabolite repression since the addition of cyclic AMP did not relieve the repressive effect of glucose (Esteban *et al.*, 1984a,b). There is currently little information on the molecular mechanism of glucose repression in *Bacillus*. However, some insight is being gained from the study of amylase mutants of *B. subtilis* that are resistant to glucose repression (Nicholson and Chambliss, 1985, 1986). One of these mutants is altered specifically in the synthesis of amylase by virtue of a point mutation that maps to *amyR*. The mutant is *cis*-acting and lies in a region, the nucleotide sequence of which is similar to that of the *lac* operator (Chambliss, personal communication). Thus this mutant seems to define a promoter region that is the recognition site for a protein involved in catabolite repression. It is particularly interesting that these mutants have finally clarified the distinction between repression of enzyme synthesis due to glucose and repression as a function of the growth cycle. Although these mutants do not respond to glucose repression, they still repress amylase synthesis during exponential growth showing that catabolite repression and temporal regulation are the results of independent molecular mechanisms (Nicholson and Chambliss, 1986).

Protease synthesis in *Bacillus* is generally susceptible to nitrogen repression in addition to catabolite repression. The extracellular proteases are usually synthesized constitutively but repressed by high concentrations of ammonium or amino acids (Doi, 1972). A variety of peptides and amino acids repress protease synthesis in the different species and, in continuous cultures of *B. licheniformis*, protease synthesis only occurs under nitrogen-limiting conditions. The molecular mechanisms are not understood.

2.4.3. Induction of Extracellular Enzymes

Many extracellular enzymes are constitutively expressed, including the industrially important amylases and proteases of *B. amyloliquefaciens* and *B. licheniformis*, but several are also inducible. Notable inducible extracellular enzymes of *Bacillus* include the amylases of some strains of *B. licheniformis* and *B. stearothermophilus* that are induced by malto-oligosaccharides, the β -amylase of *B. polymyxa* which is only synthesized in the presence of starch or a derivative of starch, and the β -glucanases of *B. circulans* (reviewed by Priest, 1977).

Efficient control of induction of an extracellular enzyme is more complicated than that of a cytoplasmic enzyme since the organism must have some means of detecting the macromolecule outside the cell. Moreover, the organism must be able to monitor the activity of the secreted enzyme in what may be a very harsh environment so that it can be synthesized at an appropriate rate. It appears that most microorganisms solve these problems by secreting enzymes at a low or intermediate basal rate. Assuming the environment is conducive, the enzyme will hydrolyze its substrate and release low molecular mass products which, when assimilated by the bacterium, will induce further enzyme synthesis (Tsuyumu, 1979). Wasteful enzyme synthesis is avoided since the products accumulate outside, and subsequently inside, the cell and restrict synthesis by catabolite and/or nitrogen repression. This

model accounts for inducible amylase synthesis in a strain of *B. licheniformis* and pectate lyase synthesis in *Erwinia carotovora*, which is the most extensively studied bacterial system (Tsuyumu, 1979).

2.5. Genetic Improvement of Producer Strains

Strain development programs are undergoing a revolution with the introduction of gene-cloning techniques for *Bacillus* (see Chapter 6). The traditional mutation and screening approach has been extremely successful and combined with gene transfer by transformation (see Chapter 4) has resulted in very high yields of amylase from *B. subtilis* (Yoneda, 1982). Although somewhat outdated, this approach should be noted since for some systems, such as the multitude of yeast cell wall lytic enzymes secreted by *B. circulans* WL12 (Estaban *et al.*, 1984a), gene cloning may not be straightforward.

The mutation program adopted by Yoneda and his colleagues involved traditional mutagenesis followed by screening for large zones of amylase hydrolysis on starch nutrient plates. Although there is no direct selection for hyperproduction of α -amylase, resistance to antibiotics that interfere with the cell envelope, such as tunicamycin and cycloserine, has proved useful for obtaining pleiotropic mutants that overproduced amylase, presumably by affecting enzyme secretion. Similarly, RNA polymerase and ribosomal antibiotics have been used to search for pleiotropic mutants (Eveleigh and Montenecourt, 1979). The success of Yoneda's approach was not fully realized until mutations of different alleles were combined in a single recipient by transformation. The mutations were not simply additive and the yield of amylase was increased 1500-fold over the wild type.

However, with current genetic technology, simply cloning an α -amylase gene into a multicopy plasmid can increase yields substantially (Palva, 1982) and for single-gene products, such as enzymes, specialized expression vectors (see Chapter 5) are the preferred route to increased yield.

3. ANTIBIOTICS

3.1. Structure and Activity

There are four general classes of antibiotic produced by *Bacillus*: (1) cyclic oligopeptides, such as bacitracin, that inhibit cell wall synthesis; (2) linear or cyclic oligopeptides, such as gramicidins and polymyxin, that interfere with membrane function; (3) basic peptides, such as the edeines, that inhibit the formation of the initiation complex on the small ribosome subunit; and (4) aminoglycoside antibiotics that affect ribosome function (Shoji, 1978).

3.1.1. Bacitracin

Derived from *B. licheniformis*, several bacitracins have been isolated but bacitracin A has the highest antibacterial activity and predominates in commercial

preparations. It comprises a linear chain of eight L- and four D-amino acids linked to a heptapeptide ring which circularizes through the ϵ -amino group of an L-Lys residue and the α -carboxyl of the C-terminal L-Asp. There is also a thiazoline ring involving the N-terminal Ile and Cys residues. Bacitracin interferes with cell wall synthesis and is active primarily against gram-positive bacteria (Katz and Demain, 1977).

3.1.2. Membrane Active Peptide Antibiotics

Tyrothricin was identified in *B. brevis* and subsequently resolved into two fractions, the tyrocidines and linear gramicidins. Tyrocidines are cyclic decapeptides and, although there is some variation in composition in tyrocidines A, B, C, and D, all contain the sequence L-Val-L-Orn-L-Leu-D-Phe-L-Pro. The cyclic structure is essential for high antibacterial activity which is directed against many gram-positive and some gram-negative bacteria although the latter are far less sensitive. One to 100 $\mu\text{g/ml}$ tyrocidine will kill *Staphylococcus aureus* but some strains of *E. coli* are not killed by as much as 1 mg/ml.

Gramicidins are also produced by *B. brevis* strains such as ATCC 8185 (Dubos) or ATCC 10068. Originally termed gramicidin D (Dubos), these linear peptides of 15 amino acid residues have now been resolved into three types: valine gramicidin A, B, and C and the isoleucine versions A, B, and C (Katz and Demain, 1977). Linear gramicidins act as ionophores and are bacteriostatic, primarily against gram-positive bacteria at concentrations of 1–30 $\mu\text{g/ml}$.

Strains of *B. brevis* (e.g., ATCC 9999 and Nagano) that do not produce tyrocidines produce gramicidin S, a cyclic peptide comprising two identical pentapeptides: L-Val-L-Orn-L-Leu-D-Phe-L-Pro. Like many peptide antibiotics, it contains amino acids not normally found in proteins such as L-Orn and D-Phe. This antibiotic like the tyrocidines has surfactant properties and is most active against gram-positive bacteria.

Polymixins, colistins, and the structurally related circulins are derived from *B. polymyxa*, "*B. colistinus*", and *B. circulans*, respectively. These antibiotics are branched cyclic decapeptides linked to a fatty acid residue. Due to high nephrotoxicity only polymyxin B, D, and E as sulfates are used clinically. They have practically no effect on gram-positive bacteria but most gram-negative bacteria are inhibited by low concentrations (0.02–5 $\mu\text{g/ml}$) of these antibiotics. They behave as cationic surface-active compounds and destroy membrane integrity.

3.1.3. Edeines

These linear oligopeptides are produced by *B. brevis* strain Vm4. They comprise β -tyrosine- β -serine- α , β -diaminopropionic acid-2,6-diamino-7-hydroxyazalaic acid-L-glycine. The L-Gly residue is attached to spermidine in edeine A and guanyl supermidine in edeine B. They have a broad spectrum of activity and inhibit both gram-positive and gram-negative bacteria, fungi, and yeasts as well as some neoplastic cells in tissue culture. In vivo, edeine inhibits initiation of protein synthesis on the 30S ribosomal subunit of prokaryotes.

3.1.4. Aminoglycoside Antibiotics

Usually associated with actinomycetes, many aminoglycoside antibiotics are now attributed to *Bacillus* of which the butirosin complex is the most studied (De Furia and Claridge, 1976). Butirosins A and B comprise neamine moieties having the aminocyclitol unit substituted at the 5' position with ribose or xylose, respectively. Although the producer organisms have been identified as *B. circulans*, typical strains of this species do not produce these compounds.

3.2. Biosynthesis of Peptide Antibiotics

Cell-free systems for the synthesis of gramicidin S, tyrocidine, linear gramicidin, edeine, bacitracin, and colistin based on a soluble cell extract, Mg^{2+} ATP, a reducing agent, and the relevant amino acids are resistant to ribosome inhibitors DNAase and RNAase and do not employ normal protein-synthesizing machinery (Katz and Demain, 1977). Many of the synthetases have been purified; gramicidin S synthetase comprises two complementary enzymes, and tyrocidine and bacitracin synthetases three complementary enzymes each (Kleinkauf and von Dohren, 1983). The best-understood system is that for gramicidin S, which has been described in detail elsewhere (Vandamme, 1981, 1983; Zimmer *et al.*, 1979). In each case synthesis is based on the multienzyme thiotemplate system in which the final sequence of amino acid residues in the peptide is determined by their positions in the enzymes bound as thioesters (Zimmer *et al.*, 1979).

3.3. Involvement of Antibiotics in Sporulation

Like many of the extracellular enzymes of *Bacillus*, antibiotics are generally synthesized as the culture enters stationary phase and the early stages of sporulation. Indeed, antibiotic production is used as a phenotypic marker for the early events of spore formation (Piggot and Coote, 1976). There have been many attempts to assign functional roles in sporulation to antibiotics but the regular isolation of antibiotic-deficient mutants that sporulate normally excludes an essential role in sporulation. Nevertheless, both tyrocidine and linear gramicidins inhibit RNA polymerase *in vitro*, gramicidin by acting on the RNA polymerase-DNA complex (Sarkar *et al.*, 1977), and tyrocidine as a result of complex formation between DNA and the antibiotic. It has been suggested that this could alter transcriptional specificity and the antibiotics would therefore be involved in sporulation (Ristow, 1977). However, mutants deficient in the production of peptide antibiotics such as linear gramicidin and gramicidin S sporulate and produce spores with normal heat resistance (Piret and Demain, 1983). Similarly, tyrocidine-negative mutants of *B. brevis* (Symons and Hodgson, 1982), bacitracin-negative mutants of *B. licheniformis*, and mycobacillin-negative mutants of *B. subtilis* (reviewed by Katz and Demain, 1977) all sporulate normally.

An essential role for these molecules in sporulation is therefore excluded (but see Modest *et al.*, 1984) and it seems that antibiotic synthesis, like the production of exoenzymes, is a response to the same environmental conditions that derepress sporulation (Ochi and Ohsawa, 1984) and may be produced to kill or inhibit other

microorganisms thereby providing a competitive advantage to the producing strain.

4. INSECT PATHOGENESIS

Although a great many bacteria cause diseases of insects, only a few are used commercially as control agents. Insect pathogenic bacteria of the genus can be assigned to five species: *B. larvae*, *B. lentimorbus*, *B. popilliae*, *B. sphaericus*, and *B. thuringiensis*. Of these *B. larvae* is a pathogen of honey bees and is avoided rather than encouraged. *Bacillus sphaericus* was regarded as a saprophyte until relatively recently when the pathogenicity of some strains for mosquito larvae was noted (Kellen *et al.*, 1965; Singer, 1973). *Bacillus thuringiensis* and *B. popilliae* are among the most successful of all microbial control agents and are good examples of the two principal approaches to the control of insect pests (Deacon, 1983). *Bacillus thuringiensis* is used as a pesticide in the same way as a chemical control agent would be used. Repeated applications are required since neither the toxin nor the spores persist in the environment for long periods. On the other hand, when *B. popilliae* is introduced into an environment from which it was previously absent, it gives a lasting chronic infection of the offending insect that should proliferate and spread throughout the population.

Microbial control of insects offers the advantages that the materials are safe compared with the more toxic chemical control agents. They have relatively slight effects on the ecological balance of the environment and they are often compatible with other agents including chemicals (Deacon, 1983). It is therefore not surprising that there is a great deal of interest in this topic, particularly with regard to insect vectors of human and animal diseases.

4.1. *Bacillus thuringiensis*

4.1.1. Classification

Bacillus thuringiensis is a close relative of *B. cereus* with which it shares considerable DNA homology (reviewed by Priest, 1981). Sometimes considered as a variety of *B. cereus* (Gordon *et al.*, 1973), it is given species status largely because of its importance as an insect pathogen. It can be readily recognized by the presence of the cytoplasmic parasporal body or crystal protein. The classification of *B. thuringiensis* is based on biochemical and serological criteria (Table II; de Barjac, 1982). Flagellar or H antigens provide 21 serotypes which are further divided by antigenic subfactors. Each type or subtype corresponds to a group of phenotypic characters and is considered a subspecies, variety, or serovar (de Barjac, 1981). With the exceptions of subspecies *israelensis*, *darmstadiensis*, and *kyushiensis*, these are principally pathogens of lepidopterous larvae. Although in some cases (e.g., subspecies *kurstaki*) there is a good correlation between serology and activity spectrum (Yamamoto, 1983), within the same serotype strains often react quite differently toward particular insect species (de Barjac, 1981). Since toxin genes are often located on plasmids (see Section 4.1.3) and these can be transmitted between two

Table II. Classification of *Bacillus thuringiensis*^a

Serotype	Subspecies	Serotype	Subspecies
1	<i>thuringiensis</i>	11a, 11b	<i>toumanoffi</i>
2	<i>finitimus</i>	11a, 11c	<i>kyushuensis</i>
3a	<i>alesti</i>	12	<i>thompsoni</i>
3a, 3b	<i>kurstaki</i>	13	<i>pakistani</i>
4a, 4b	<i>sotto</i>	14	<i>israelensis</i>
4a, 4b	<i>dendrolimus</i>	15	<i>indiana</i>
4a, 4c	<i>kenyae</i>	16	<i>dakota</i>
5a, 5b	<i>galleriae</i>	17	<i>tohokuensis</i>
5a, 5c	<i>canadensis</i>	18	<i>kumaotoensis</i>
6	<i>subtoxicus</i>	19	<i>tochigiensis</i>
6	<i>entomocidus</i>	20	<i>yunnanensis</i>
7	<i>aizawai</i>	21	<i>colmeri</i>
8a, 8b	<i>morrisoni</i>	— ^b	<i>wuhanensis</i>
8a, 8c	<i>ostriniae</i>	— ^b	<i>fowleri</i>
9	<i>tolworthi</i>	— ^c	<i>galechae</i>
10	<i>darmstadiensis</i>	—	

^aAdapted from Dean (1985). For full bibliography, see Dean (1985).

^bNo flagella, cannot be serotyped.

^cRecently classified as H-serotype 1.

strains in a mixed culture (Gonzalez *et al.*, 1982), it is likely that a strain can change the characters of its toxin by acquiring a new plasmid. Serotypes 10, 11a, 11c, and 14 (Table II) are specifically pathogenic to mosquito and *Simuliidae* (black fly) larvae (Goldberg and Margalit, 1977). This is particularly exciting since it offers the possibility of microbiological control of insect vectors of human disease, in particular the yellow fever mosquito *Aedes aegypti*, which is a vector of a large number of diseases including yellow fever, dengues, chickunguaya, and various encephalites. Finally, some recently isolated strains are pathogenic for coleoptera and there is no reason why strains for other insects should not exist.

4.1.2. Pathogenicity

Bacillus thuringiensis itself is only mildly pathogenic but 2–3 hr after the end of exponential growth, i.e., after completion of the forespore septum, it produces the crystal protein. The microbial insecticide comprises spores and crystals which, when ingested by larvae, cause gut paralysis, probably by upsetting the ionic balance of the gut. The spore survives its passage through the gut, penetrates the weakened midgut wall, and multiplies in the hemolymph. Death results from either intoxication or septicemia (Aronson *et al.*, 1986).

The crystal is a protoxin with a molecular mass of about 130,000 (Huber *et al.*, 1981). It passes unchanged through the acidic stomachs of most insects; however, many lepidoptera have an alkaline midgut, and under these conditions proteolysis by the insect releases a protease-resistant peptide with a molecular mass of about 65,000–85,000, i.e., the δ -endotoxin (Huber *et al.*, 1981; Chestukhina *et al.*, 1982). Differences in peptide maps of the protoxins derived by high-performance liquid

chromatography (HPLC) appear to correlate with differences in the spectrum of activity (Yamamoto, 1983). There is also some evidence that the proteolytic enzymes of different insects release different polypeptides from the crystals, increasing further the specificity that might occur (Deacon, 1983).

Some insecticidal strains produce a second toxin—the β -exotoxin—which has a wider spectrum of activity. This is an ATP analog and is extremely toxic to a range of insects and also to some vertebrates. The strains used commercially do not produce β -exotoxin and are without harmful effects on vertebrates.

Bacillus thuringiensis subspecies *israelensis* (serotype 14) also produces a crystal toxin the insecticidal activity of which is centered on the larval midgut. This protein differs from the lepidopterous-active crystals in size, appearance, antigenicity, and subunit composition (Huber *et al.*, 1981). Analysis by HPLC revealed three types of crystal patterns in strains of *B. thuringiensis* (Yamamoto, 1983). The first (type A) showed two protein peaks of which P-1 accounted for about 95% of the protein. This pattern was detected in strains of subspecies *thuringiensis*, subspecies *kurstaki*, subspecies *kenyae*, and subspecies *tolworthi*. Type B contained only the P-1 peak and type C, represented by subspecies *israelensis*, contained only the second peak (P-2). Interestingly, some strains of subspecies *finitimus*, subspecies *kysheunsi*, subspecies *thompsoni*, and subspecies *indiana* also gave type C patterns. A survey of strains for the presence of P-2 revealed that all P-2-producing strains have been reported to possess mosquitocidal activity (Hall *et al.*, 1977; Yamamoto, 1983). Thus pathogenesis to mosquitoes and blackflies is not restricted to subspecies *israelensis*.

4.1.3. Location of Crystal Protein Genes

Since strains of *B. thuringiensis* frequently lose the ability to produce crystal proteins and rarely if ever revert to crystal production (Cry⁺), it seemed likely that the genes responsible might be located on plasmids (Debabov *et al.*, 1977; Stahly *et al.*, 1978). Three approaches have been adopted to examine this possibility.

Plasmid-curing studies have indicated that large (50–105 MDa) plasmids code for crystal genes in strains HD-L and HD-73 (subspecies *kurstaki*) and HD-2 (subspecies *thuringiensis*), since loss of these plasmids leads to loss of crystal production (Gonzalez *et al.*, 1981). Similarly, in subspecies *israelensis* the protoxin gene is located on a large (75-MDa) plasmid (Sekar and Carlton, 1985). It should be noted that most plasmid detection techniques are not suitable for detecting these large (>60-MDa) plasmids of *B. thuringiensis* and only careful application of the Eckhardt (1978) procedure resolves all of these molecules (Dean *et al.*, 1982).

The second approach involves a recently discovered mating process. By mixing two strains of *B. thuringiensis* or *B. thuringiensis* and *B. cereus* together, plasmid transfer at high frequency can be obtained (Gonzalez *et al.*, 1982). In this way, Cry⁻ strains were converted to Cry⁺ and the plasmid recipients made crystals of the same antigenicity as the donor rather than recipient, implying that the structural gene resided on the plasmid.

Finally, cloning of the crystal protein genes has allowed the development of hybridization probes for crystal genes. Crystal protein genes have been cloned from strains HD-1 (subspecies *kurstaki*; Schnepf and Whiteley, 1981; Whiteley *et al.*, 1982; Held *et al.*, 1982), a strain of subspecies *thuringiensis* (Klier *et al.*, 1982) and subspecies *israelensis* (Sekar and Carlton, 1985) among others (reviewed by Aronson *et*

al., 1986). Kronstad *et al.* (1983) developed a gene-specific hybridization probe to demonstrate the plasmid location of the crystal protein gene in 17 strains representing nine different subspecies. In eight of the 17 strains a single plasmid hybridized with the probe and in seven strains more than one plasmid hybridized. In two strains plasmid resolution was too poor to assign the gene to a specific plasmid and in only one strain (subspecies *wuhanensis*) was the gene probably located on the chromosome. This conflicts with other hybridization studies where chromosomal and plasmid locations were implicated for the gene in subspecies *kurstaki* (Held *et al.*, 1982) and subspecies *thuringiensis* (Klier *et al.*, 1982).

4.1.4. Regulation of Crystal Protein Synthesis

Since the crystal protein is synthesized during the early stages of sporulation, it has been suggested that sporulation-specific forms of RNA polymerase may be involved. Analysis of the promoter and mRNA initiation sites of the gene from subspecies *kurstaki* (HD-1) supports this contention and shows that crystal protein synthesis is under transcriptional control from a promoter that resembles the σ^{37} promoters of *B. subtilis* (Wong *et al.*, 1983). A role for cryptic plasmids in regulating protoxin synthesis has also been implied from plasmid transfer experiments which show that such plasmids can influence the amount of protoxin synthesized by recipient strains. They do not, however, alter the time of appearance of the protein (Minnich and Aronson, 1984).

4.2. *Bacillus sphaericus*

It is often forgotten that some strains of *B. sphaericus* are insecticidal. *Bacillus sphaericus* compares well with *B. thuringiensis* subspecies *israelensis* and, while the former is more active on certain *Aedes* species, *B. sphaericus* is more active against certain *Anopheles* species. Pathogenic strains of *B. sphaericus* differ from non-pathogenic strains in DNA sequence homology (Krych *et al.*, 1980). They constitute several serological (de Barjac *et al.*, 1980) and phage-typing groups (Yousten *et al.*, 1980), and many strains with good potential exist (Singer, 1980, 1981).

Crystal-like parasporal inclusions have been identified in some but not all strains of *B. sphaericus* (Davidson and Myers, 1981), and although the toxin has also been reported to be associated with the spore itself and the cell wall and membrane of prespore cells, its main activity is associated with these inclusion bodies (Davidson, 1984). The toxin affects the larval gut and death may occur from 4 to 48 hr after ingestion depending on the dose. Plasmids are present in toxigenic strains but their relationship to toxin production is unclear (Davidson, 1984). Successful field trial applications suggest that strains of *B. sphaericus*, like those of *B. thuringiensis*, may be a valuable alternative to chemical insecticides in the near future (Lacey, 1984).

4.3. *Bacillus popilliae*

There are two principal agents of the milky disease of scarabaeid beetles: *B. popilliae* and *B. lentimorbus*. Their taxonomy is confused; some consider *B. lentimor-*

bus to be a variety of *B. popilliae* but others consider the two to be sufficiently different as to warrant separate species status (Gordon *et al.*, 1973; Bulla *et al.*, 1978). They differ in that sporulating cultures of *B. popilliae* contain a parasporal crystalline body while those of *B. lentimorbus* do not. Other strains isolated from milky disease-infected beetles, such as "*B. fribourgensis*" and "*B. euloomarhae*," are probably synonyms of varieties of *B. popilliae*.

These are fastidious organisms that do not grow on nutrient agar. Methods for their cultivation are described by Bulla *et al.* (1978). They do not sporulate readily in artificial media although *in vivo* sporulation is very efficient. Good *in vitro* sporulation of *B. larvae* (a pathogen for honeybee larvae) was recently reported by Dingman and Stahley (1983). Oxygen toxicity in these essentially catalase-negative bacteria (Digman and Stahley, 1984), as well as different modes of sugar metabolism *in vitro* and in the diseased host, are thought to be responsible for the inhibition of sporulation in artificial media. Comprehensive reviews of *B. popilliae* were published recently (Bulla *et al.*, 1978; Milner, 1981).

Spores of *B. popilliae* are ingested by the larvae, germinate in the gut, and begin to multiply. The bacteria invade into the hemolymph and by about 10 days a typical milky appearance is apparent due to the massive numbers of bacteria. Sporulation ensues and at the time of death about 5×10^9 spores are released (Deacon, 1983). The crystal does not seem to be involved in toxicity but this has not been entirely ruled out.

Bacillus popilliae has been used in the United States for the control of the Japanese beetle (*Popillia japonica*) for several decades. The spores are more resistant to environmental factors than those of *B. thuringiensis* and, once introduced into an area, persist for very long periods. Although very successful in reducing the overall number of Japanese beetles over the past 40 years, the spore preparations can only be produced by costly *in vivo* methods. The capability of producing spores *in vitro* will be a most valuable development.

5. PURINE NUCLEOSIDES

Purine nucleotides and nucleosides have found applications in medicine but by far their major use is as flavor enhancers in foods. Guanylic acid (GMP), inosinic acid (IMP), and xanthanylic acid (XMP) exhibit taste enhancement, and both GMP and IMP are produced on a large scale in Japan. Although the direct production of nucleotides from *Corynebacterium glutamicum* and related bacteria is possible, the preferred approach is to use mutants of *B. subtilis* to produce the respective nucleosides with subsequent chemical phosphorylation (Demain, 1978).

The biochemistry of purine ribonucleotide biosynthesis is virtually identical in all living systems and has been fully reviewed by Demain (1978). First, there is a complex feedback inhibition and end-product repression of enzyme synthesis that governs purine nucleotide biosynthesis in *B. subtilis*. By modifying various components of this pathway, overproduction of individual nucleosides can be achieved. Resistance to toxic purine analogs results in constitutive mutants that are no longer sensitive to feedback inhibition and/or end-product repression. Second, purine auxotrophs can be fed growth-limiting concentrations of their requirements in order to lower intracellular levels of inhibitory metabolites. For example, all inosine-

producing strains are adenine auxotrophs which lack the ability to synthesize adenylosuccinate synthetase (Ishii and Shiio, 1973). This relieves the feedback inhibition of phosphoribosyl pyrophosphate amidotransferase by AMP and leads to an increased flux in the IMP:GMP pathways (Fig. 1a,b). (The amount of adenine in the medium is kept low so that high intracellular levels of AMP do not accumulate.) Because of the repression of IMP dehydrogenase by GMP this results in accumulation of inosine. To relieve the repression by GMP of enzymes of the common pathway, IMP dehydrogenase-deficient (xanthine-requiring) mutants were isolated which had an enhanced yield (Fig. 1c). Finally, AMP repression was eliminated by

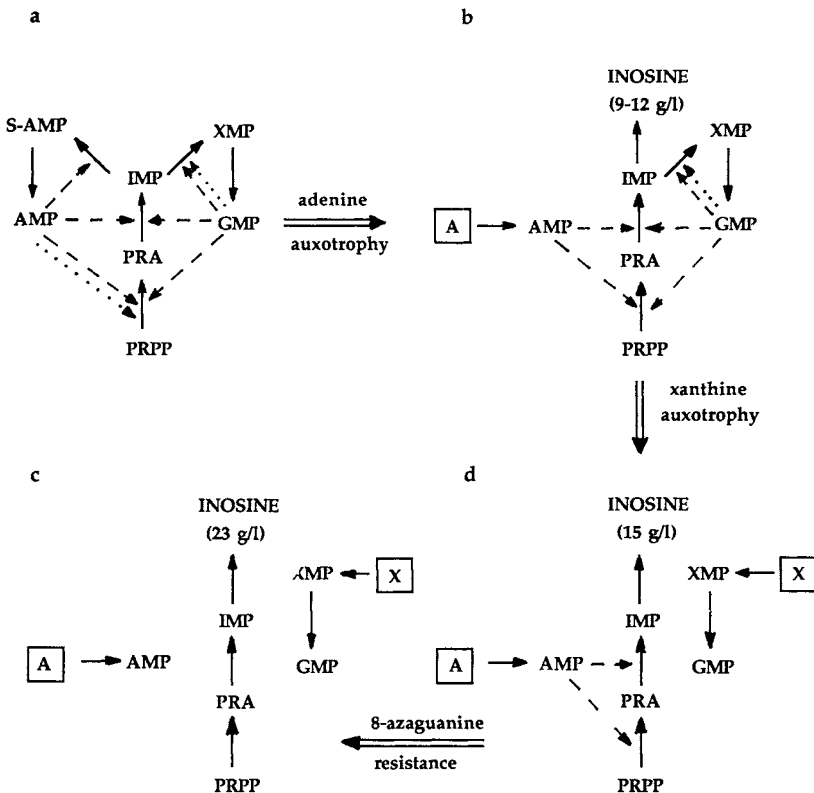


Figure 1. Diagram showing the development of the inosine fermentation using *B. subtilis* (adapted from Demain, 1978). The metabolic pathways are shown by solid arrows, repression of enzyme synthesis by dashed arrows, and dotted arrows indicate feedback inhibition of enzyme activity. (a) The initial strain which lacked AMP deaminase and GMP reductase. (b) Adenine auxotroph lacking adenylosuccinate synthase accumulates inosine but requires adenine (boxed). (c) Xanthine auxotroph lacking IMP dehydrogenase loses GMP repression of the main metabolic route and excretes more inosine. (d) Elimination of AMP repression of the metabolic pathway achieved by mutating to 8-azaguanine resistance. PRPP, phosphoribosyl pyrophosphate; PRA, phosphoribosylamine; S-AMP, adenylosuccinate; AMP, GMP, IMP, and XMP, adenosine, guanosine, inosine, and xanthosine monophosphates.

selecting for resistance to 8-azaguanine and, by combining the various mutations, 23 g/liter inosine could be produced (Fig. 1d). Similar combinations have been used to develop adenosine, xanthosine, and guanosine fermentations, which have been reviewed by Demain (1978).

6. MISCELLANEOUS USES OF BACILLI

6.1. Immobilization of Enzymes and Whole Cells

One of the major industrial applications of an immobilized enzyme is the use of glucose isomerase to convert glucose derived from starch into high-fructose syrups. Since glucose isomerases are invariably intracellular enzymes, the expensive extraction, purification, and immobilization of the enzyme can be avoided by using immobilized whole cells. The most widely used preparation is that from Novo, in which *B. coagulans* cells are broken to allow diffusion of substrate and product while the cellular material is crosslinked using glutaraldehyde. The cells are then packed in cylinders for use in column reactors (Bucke, 1983).

A promising field for the application of immobilized enzymes is the cell-free synthesis of peptide antibiotics. Although the approach has not been entirely successful to date, model systems for gramicidin S synthesis are pointing the way. Using the two gramicidin S synthetases, gram quantities of the antibiotic have been prepared (Demain *et al.*, 1976) but the successful operation of continuous reactors requires the regeneration of ATP, which has proved to be the major obstacle (Vandamme, 1983).

Immobilized living cells offer advantages over traditional fermentations of both continuous production over long periods and high product output. Since many of the products of *Bacillus* are associated with postexponential phase cells, they might be produced efficiently by growth-suppressed, immobilized cells. α -Amylase production by *B. subtilis* immobilized in polyacrylamide and carrageenan (Kokubu *et al.*, 1978; Suzuki and Karube, 1979) and *B. amyloliquefaciens* (Shinmyo *et al.*, 1982) has been achieved on a laboratory scale but diffusibility remains a problem for these aerobic processes. Bacitracin has also been produced by immobilized cells (Suzuki and Karube, 1979; Morikawa *et al.*, 1980). Of particular note is the use of immobilized *B. subtilis* for the continuous synthesis and secretion of insulin using the rat gene cloned into a secretion vector based on the penicillinase gene from *B. licheniformis* (Mosback *et al.*, 1983).

6.2. Surface-Active Agents

Biodegradable surfactants of microbial origin are currently achieving wide attention (Cooper and Zajic, 1980). Most of the common examples are glycolipids derived from coryneform bacteria grown on hydrocarbons or related substances, but there are advantages in growing the producer organism on cheap, renewable resources such as starch. The lipopeptide surfactin from *B. subtilis* is synthesized in aqueous media and has exceptional surface activity. It lowers the surface tension of

water considerably more than any other biosurfactant (Cooper *et al.*, 1981). It comprises a cyclic lipopeptide containing a carboxylic acid and seven amino acids (Kakinuma *et al.*, 1969). Its ability to lyse erythrocytes has been exploited to develop a simple screen for surfactant production based on sheep blood agar. In an initial screen of 19 isolates, various unidentified bacteria produced promising surfactant activity (Mulligan *et al.*, 1984).

6.3. Other Uses

The list of possible applications of *Bacillus* is long and increases at a rapid rate. In general this genus is favored for industrial purposes because it is largely non-pathogenic, grows quickly on inexpensive media, and, in the case of *B. subtilis*, is genetically and physiologically well studied. Thus, in addition to the exclusive uses of *Bacillus*, such as for insecticides, there is considerable interest in using *Bacillus* as an alternative producer of established products. Some possibilities include protein production (Tsukagoshi *et al.*, 1984), steroid conversions, and riboflavin production (Debabov, 1982). Although the manufacture of amino acids is dominated by coryneform bacteria, there is scope for the use of *Bacillus* in this respect. There are two aspects of the genetic improvement of amino acid-producing microorganisms. The biosynthetic pathways can be affected by the removal of end-product repression and feedback inhibition, usually by the development of toxic analog resistance (e.g., Kisumi *et al.*, 1971). However, permeability is also an important factor and L-glutamic acid production by the original *Corynebacterium glutamicum* was found to be largely due to permeability changes induced by growth on a limiting supply of biotin. Since many strains of *Bacillus* produce extracellular amino acids naturally, it seems likely that permeability may not be a serious problem in these bacteria. Moreover, Debabov (1982) argued that the relatively simple regulation of amino acid pathways in *Bacillus* might indicate that it would be more simple to interfere with and increase yields of amino acids in these bacteria than others. However, at present only L-tryptophan and L-arginine are produced by member of this genus at levels approaching economic feasibility (Kinoshita and Nakayama, 1978).

The metabolic versatility of *Bacillus* is being examined for the biodegradation of wastes and pesticides and for polysaccharide and riboflavin production. Most of the literature covering these areas is in the form of patents and has been briefly reviewed by Debabov (1982).

7. FUTURE FOR BACILLI

New products and applications within the current usage of *Bacillus* are being and will continue to be discovered. In the case of enzymes for use in foods, the safety testing required before a new product can be launched is extremely expensive but this has not prohibited the introduction of pullulanase from "*B. acidopullulyticus*," and other enzymes of similar potential will undoubtedly be developed. However, a rapidly expanding market exists for relatively low-volume, high-cost enzymes for use in diagnostic kits and biosensors. Robust, stable enzymes are

needed and it is surprising that the thermophilic bacilli in particular have not been examined to a significant extent for such enzymes.

Similarly, there are undoubtedly new strains pathogenic for insects that have yet to be isolated. It has been reported that some strains within the *B. brevis*/*B. circulans* complex are insect pathogens (Singer, 1981), but the poor taxonomy of these bacteria is hampering their exploitation. Random screening for pathogenicity against a variety of insects is time consuming and expensive, and a phenotypic data base that would allow isolation and identification of potential pathogens would assist the process enormously (see Chapter 3).

However, rather than screening for new enzymes, it seems likely that protein engineering may provide the desirable properties in existing enzymes by site-directed mutagenesis. Some prediction of the types of amino acid substitution required for particular purposes can be obtained from structure analysis and molecular modeling but this is not always effective. For example, disulfide bridges are usually associated with improved stability of enzymes but when two bridges were introduced into subtilisin (from *B. amyloliquefaciens*) at positions 22-87 and 24-87 one had no effect on stability and the second actually rendered the molecule more susceptible to autolysis (Wells and Powers, 1986). However, a predictable change in the pH dependence of the enzyme was achieved by altering the overall surface charge by substituting Asp 99 by Ser at a position that is some 15 Å from the active site (Thomas *et al.*, 1985). Moreover, mutants with greatly enhanced resistance to oxidation have been obtained by mutating Met 222 into a nonoxidizable amino acid such as Ser, Ala, or Leu (Estell *et al.*, 1985). So the future for engineered proteins seems very bright, particularly in situations where well-characterized molecules such as subtilisin are involved.

It seems likely that some of the major advances in the exploitation of *Bacillus* will not come about through the expansion of existing and new products but rather through the utilization of *B. subtilis* and related bacteria for new technologies. Gene cloning in *B. subtilis* is now at an advanced stage (see Chapter 6) and has the advantage over *E. coli* that secretion of the product is more readily obtainable. Combined with the ability to block extracellular protease synthesis (Stahl and Ferrari, 1984), high yields of relatively pure product are now possible and immobilized systems are being developed with a view to continuous production (Mosbach *et al.*, 1983).

Although many of the antibiotics of bacilli are of limited clinical value, the relatively recent discovery of aminoglycoside antibiotics has widened their scope. Furthermore, the metabolic pathways involved might be used for the manufacture of clinically valuable antibiotics. For example, amikacin is currently being produced from kanamycin by a chemical process but can be manufactured more economically by feeding butirosin-producing strains of *B. circulans* with kanamycin (Thompson and Davis, 1984).

The crystal proteins of *B. thuringiensis* represent an unusual channeling of protein synthesis, since about 30% of the cell's protein synthetic activity at the time of formation must be devoted to these particular peptides. This is similar to that of the most finely tuned expression vectors of *E. coli* and it is possible that it could be further increased by genetic manipulation. It may be that *B. thuringiensis* will be popular in the next generation of industrial hosts for cloned genes if such high yields can be realized.

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Appendixes

A Selection of Culture Collections from Which *Bacillus* Strains May Be Obtained

I

DIETER CLAUS and DAGMAR FRITZE

Country	Culture collection organization	Acronym
Australia	Department of Microbiology University of Queensland St. Lucia 4067, Queensland	UQM
Belgium	Laboratorium voor Microbiologie en Microbiele Genetica Faculteit der Wetenschappen K.L. Ledeganckstraat 35 B-9000 GENT	LMG
Bulgaria	National Bank for Industrial Micro- organisms and Cellular Cultures Sofia—1113 Lenin Bd 125 bl 2 TCHAPAEV 55	NBIMCC
China	China Committee for Culture Col- lections of Microorganisms Chinese Academy of Sciences Institute for Microbiology Zhongguancon, Beijing	CCCCM
Czechoslovakia	Czechoslovak Collection of Microorganisms J.E. Purkynje University Trida Obrancu Miru 10 662 43 BRNO	CCM

DIETER CLAUS and DAGMAR FRITZE • German Collection of Microorganisms and Cell Cultures, 3300 Braunschweig, Federal Republic of Germany.

Country	Culture collection organization	Acronym
Denmark	Bacillus Collection Institute of Hygiene University of Aarhus Universitetsparken Building 161 DK-8000 Aarhus, Jylland	BCIH
Finland	VTT Collection of Industrial Micro-organisms VTT, Biotechnical Laboratory Technical Research Centre of Finland Tietotie 2 SF-02150 ESPOO	VTT
France	Collection National de Cultures de Microorganismes Institut Pasteur 25 rue du Docteur Roux F-72724 Paris Cedex 15	CNCM (IDA)*
Germany, DDR	Zentralinstitut für Mikrobiologie und Experimentelle Therapie Akademie der Wissenschaften der DDR IMET-Kultursammlung Beutenbergstrasse 11 DDR-6900 Jena	IMET
Germany, FRG	DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH Mascheroder Weg 1B D-3300 Braunschweig	DSM (IDA)*
Hungary	National Collection of Agricultural Ind. Microorganisms University of Horticulture Department of Microbiology Somloisut 14-16 H-1118 Budapest	NCAIM (IDA)*
India	National Collection of Industrial Microorganisms Biochemistry Division National Chem. Lab. CSIR Poona, Maharashtra, 411-08	NCIM

Country	Culture collection organization	Acronym
Japan	Culture Collection of the Institute for Fermentation Osaka Juso-Homachi 2-17-85 Yodogawa-ku, Osaka 532	IFO
	Institute for Applied Microbiology University of Tokyo Yayoi 1-1-1 Bunkyo-ku, Tokyo 113	IAM
	Japan Collection of Microorganisms Riken, Wako-shi Saitama 351	JCM
	Fermentation Research Institute 1-3, Higashi 1-Chome Tsukuba-shi Ibaraki-ken 305	FRI (IDA)*
Netherlands	Culture Collection Laboratory of Microbiology Delft University of Technology Julianalaan 67 A Delft	LMD
Philippines	Natural Science Research Centre Culture Collection University of The Philippines Quirino and Roces Avenues Quezon City, Rizal	UPCC
Poland	Polish Collection of Microorganisms Institute of Immunology and Ex- perimental Therapy Polish Academy of Sciences Czerska 12 PL-53 114 Wroclaw	PCM
Spain	Coleccion Espanola de Cultivos Tipo Departamento de Microbiologia Facultad de Ciencias Biologicas Universidad de Valencia Burjasot, Valencia	CECT

(continued)

Country	Culture collection organization	Acronym
Sweden	Culture Collection of University of Goteborg Dept of Clinical Bacteriology Guldhedsgatan 10 S-413 46 Goteborg	CCUG
Switzerland	Centre de Collection de Type Microbien Institut de Microbiologie Universite de Lausanne 44 rue du Bugnon CH-1011 Lausanne	CHUV
Thailand	TISTR Culture Collection Thailand Institute of Scientific and Technological Research Phahonyothin Road Bangkok 9	TISTR
United Kingdom	National Collection of Industrial and Marine Bacteria Ltd. Torry Research Station P.O. Box 31 135 Abbey Road Aberdeen AB9 8DG (Scotland)	NCIMB (IDA)*
United States of America	American Type Culture Collection 12301 Parklawn Drive Rockville, Maryland 20852	ATCC (IDA)*
	ARS Culture Collection Northern Regional Research Centre Agricultural Research Service 1815 Nth University Street Peoria, Illinois	NRRL (IDA)*
	Bacillus Genetic Stock Center The Ohio State University Department of Biochemistry 484 West 12th Avenue Columbus, Ohio 43210	BGSC

Country	Culture collection organization	Acronym
Union of Soviet Socialist Republics	All-Union Collection of Microorganisms Institute of Biochemistry and Physiology of Microorganisms USSR Academy of Sciences, Puschino, Moscow Region 142292	AUCM-IBFM (IDA)*

*IDA = International Depository Authority.

Bacillus Identification Methods

II

FERGUS G. PRIEST

1–5. Morphology

Gram stain. Perform Gram stains on young (<24 hr) nutrient agar (NA) cultures.

1. Measure length of cells (</> 3 μm)
2. Gram variable is scored gram-positive.

Spore stain/phase-contrast microscopy. Determine:

3. Spore shape (oval/round)
4. Spore position (central/terminal)
5. Swelling of sporangium

6–10. Degradation tests

6. Aesculin hydrolysis

Use (g/liter):	Aesculin	1.0
	Ferric citrate	0.5
	Peptone	10.0
	NaCl	5.0
	Agar	To solidify

Sterilize 115°C 10 min, incubate for 5 days

7. Elastin hydrolysis

Use 0.3% elastin in NA. Clearing round colonies after 5 days is positive.

8. Hippurate hydrolysis

Use (g/liter):	Tryptone	10.0
	Beef extract	3.0
	Yeast extract	1.0
	Glucose	1.0
	Na ₂ HPO ₄	5.0

FERGUS G. PRIEST • Department of Brewing and Biological Sciences, Heriot–Watt University, Edinburgh EH1 1HX, United Kingdom.

Na hippurate	10.0
Adjust to pH	6.8

Incubate in tubes for 5 days. Mix 1 ml culture with 1.5 ml of 50% (v/v) H_2SO_4 . Crystal formation is positive.

9. Starch hydrolysis

Use 0.5% soluble starch in NA. Flood with iodine solution after 5 days.

10. Urease

Use Christensen's urease (Oxoid).

11–14. Antibiotic inhibition

11. Chloramphenicol	4.0 $\mu g/ml$
12. Nalidixic acid	32.0 $\mu g/ml$
13. Polymyxin	16.0 $\mu g/ml$
14. Streptomycin	8.0 $\mu g/ml$

Use indicated amounts of antibiotic in NA. Score for growth after 4 days.

15–22. Sugar fermentations

15. Cellobiose	}	All prepared at 10% w/v and filter sterilized.
16. Fructose		
17. Galactose		
18. Lactose		
19. Mannose		
20. Raffinose		
21. Salicin		
22. Xylose		

Use (g/liter)	$(NH_4)_2HPO_4$	1.0
	KCL	0.2
	$MgSO_4$	0.2
	Yeast extract	0.2
	Agar	To solidify
	Adjust to pH	7.0

After autoclaving add 15 ml of 0.04% bromocresol purple per liter and sugar at 0.5% final volume. Incubate as slopes for 5 days.

23–24. Organic acid utilization

23. Citrate

24. Succinate

Use (g/liter)	NaCl	1.0
	$MgSO_4$	0.2
	$(NH_4)_2HPO_4$	0.5

Yeast extract	0.2
Citrate/succinate	2.0 g
0.04% phenol red	20 ml
Agar	To solidify
Adjust to pH	6.8

Incubate for 4 days, red coloration is positive.

25–30. Miscellaneous tests

25. Growth at 50°C

Use NA. Incubate 2 days.

26. Growth in 10% NaCl.

Use NaCl (to 10% w/v) in NA. Incubate up to 4 days.

27. Anaerobic growth

Use (g/liter)	Tryptone	10.0
	NaCl	5.0
	Sodium thioglycollate	2.0
	Sodium formaldehyde sulfoxylate	1.0
	Agar	To solidify
	pH	7.2

(This medium is available as BBL No. 10919)

Stab inoculate tubes and inspect for growth in anaerobic portion.

28. Nitrate reduction

Use 1% (w/v) KNO_3 in nutrient broth. Test for nitrate after 3 and 5 days using A: 0.8% sulfanilic acid in 5 N acetic acid and B: 0.5% α -naphthylamine in 5 N acetic acid.

29. Oxidase

Use 1% tetramethyl phenylenediamine dihydrochloride. Purple coloration within 10 seconds is positive.

30. Voges Proskauer

Use (g/liter)	Peptone	5.0
	K_2HPO_4	5.0
	Glucose	5.0
	Adjust to pH	7.0

Use α -naphthol and KOH to test for acetoin after 3 and 5 days.

The Nucleotide Sequence and Restriction Site Analysis of pUB110 (Clockwise Relative to Fig. 1, Chapter 6, Section 3.1)

III

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10	20	30	40	50	60
<u>GAATTCCTTA</u>	GTGCTTTCAT	AGATTAAACT	CACATCACGC	<u>TTAAATCGC</u>	TTATTTTAGA
70	80	90	100	110	120
<u>CTTTAAAGAC</u>	<u>TTGTTTCTT</u>	<u>CAAGCAACTC</u>	ATTATAATCA	TTTACATTTT	CATTAAATCG
130	140	150	160	170	180
CTCTACAAGA	CCACTATATT	TTTCTTTAAC	<u>TTGCCCATGT</u>	TCTTTACTTA	ATTTTTTATA
190	200	210	220	230	240
TTCTCTCGCC	ATATCAGTAC	<u>TCATGAGATT</u>	<u>TCTAACATGC</u>	TGTTTTAACC	TATCGTTATC
250	260	270	280	290	300
<u>TCTCGCAGCA</u>	<u>GTCACTAAGT</u>	TTTTATAATC	ACGCTCCGAT	ATAACAACAT	TTTTGGTTGG
310	320	330	340	350	360
TTTCTTTTCT	GTTTTCATTA	TTTCTTTTCC	CAAACCAAAC	<u>ATGGACTTTT</u>	<u>CACCCGTTGG</u>
370	380	390	400	410	420
CACTTCAACA	<u>CTTTTCATGT</u>	GTCGTTTCGC	<u>TGGTACTTCT</u>	AAATCTGATT	TAACTTTATC
430	440	450	460	470	480
GCTATAAGCA	GTCCATTCAT	CTTTTTTAAC	TGCTAAATTT	<u>TTTTCTAGAA</u>	AATCAATCTC
490	500	510	520	530	540
TTTTTCCAAA	GTTTGTTTTT	<u>TAAATTTAGC</u>	<u>TGTCTCAATA</u>	TGTTTACGGT	CAGAGCCACG
550	560	570	580	590	600
<u>TTCAACCACGC</u>	TTCAACTCAA	AACCCTGTTT	<u>TTTCATATGC</u>	<u>TCGGGGAATT</u>	TATCTTGTAG
610	620	630	640	650	660
CCATAACAGT	TCTTGACGAT	TAAACACATT	TTTTCCTTGC	AGTTTTCAT	CACGCATAGG
670	680	690	700	710	720
CACAACACCT	<u>AAATGCATGT</u>	<u>GAGGGGTTTG</u>	CTCATCATTA	TGAAGTGTG	<u>CATAAGCAAT</u>
730	740	750	760	770	780
<u>ATTTTGCTTG</u>	CCATATCGTT	CGGAAAATAA	TTTATAACTT	<u>TCCTCAAAAA</u>	ATCGTTTTTG
790	800	810	820	830	840
<u>TTCTCCTGGA</u>	<u>TCCAGTTGCT</u>	CAAAAAAATC	TCGGTCAGAT	GTTACTAGCA	ACTCATTTAC
850	860	870	880	890	900
AAGAACAGCA	<u>TCTTTCCTCC</u>	GTTTTTCTTG	<u>TACCTGTTTT</u>	<u>TTGTGATTCA</u>	ATAATTTCTT
910	920	930	940	950	960
TGACACGTTT	GTTGTAATCA	<u>ATATTTTAT</u>	CATTTTTCAA	ATCATAATTT	TCACGTGTTT
970	980	990	1000	1010	1020
GCT <u>CATGGTC</u>	AATATCATCA	TTCGTTCTAC	TTTTTCGCTC	TCTTTGATTA	<u>TGAAATTGCA</u>
1030	1040	1050	1060	1070	1080
<u>TGCCTTTTAG</u>	<u>TCCAGCTGAT</u>	TTCACTTTTT	<u>GCATTCTACA</u>	AACTGCATAA	<u>CTCATATGTA</u>

1090	1100	1110	1120	1130	1140
AATCGCTCCT	TTTTAGGTGG	CACAAATGTG	AGGCATTTTC	GCTCTTTCCG	GCAACCACTT
1150	1160	1170	1180	1190	1200
CCAAGTAAAG	TATAACACAC	TATACTTTAT	ATTTCATAAG	TGTGTGTCCCT	GCGAGGCTGT
1210	1220	1230	1240	1250	1260
CGGCAGTGCC	GACCAAAACC	ATAAAACCTT	TAAGACCTTT	CTTTTTTTTA	CGAGAAAAAA
1270	1280	1290	1300	1310	1320
GAAACAAAAA	AACCTGCCCT	CTGCCACCTC	AGCAAAGGGG	GGTTTTGCTC	TCGTGCTCGT
1330	1340	1350	1360	1370	1380
TTAAAAATCA	GCAAGGGACA	GGTAGTATTT	TTTGAGAAGA	TCACTCAAAA	AATCTCCACC
1390	1400	1410	1420	1430	1440
TTTAAACCTT	TGCCAATTTT	TATTTTGTCC	GTTTTGTCTA	GCTTACCGAA	AGCCAGACTC
1450	1460	1470	1480	1490	1500
AGCAAGAATA	AAATTTTTAT	TGCTCTTCGG	TTTTCTAGTG	TAACGGACAA	AACCACTCAA
1510	1520	1530	1540	1550	1560
AATAAAAAAG	ATACAAGAGA	GGTCTCTCGT	ATCTTTTATT	CAGCAATCGC	GCCCCATTGC
1570	1580	1590	1600	1610	1620
TGAACAGATT	AATAATAGAT	TTTAGCTTTT	TATTTGTTGA	AAAAAGCTAA	TCAAATTGTT
1630	1640	1650	1660	1670	1680
GTCGGGATCA	ATTACTGCAA	AGTCTCGTTC	ATCCCACCAC	TGATCTTTTA	ATGATGTATT
1690	1700	1710	1720	1730	1740
GGGGTGCAAA	ATGCCCAAAG	GCTTAATATG	TTGATATAAT	TCATCAATTC	CCTCTACTTC
1750	1760	1770	1780	1790	1800
AATGCGGCAA	CTAGCAGTAC	CAGCAATAAA	CGACTCCGCA	CCTGTACAAA	CCGGTGAATC
1810	1820	1830	1840	1850	1860
ATTACTACGA	GAGCGCCAGC	TTCATCACTT	GCCTCCCATTA	GATGAATCCG	AACCTCATTA
1870	1880	1890	1900	1910	1920
CACATTAGAA	CTGCGAATCC	ATCTTCATGG	TGAACCAAAG	TGAAACCTAG	TTTATCGCAA
1930	1940	1950	1960	1970	1980
TAAAAACCTA	TACTCTTTTT	AATATCCCCG	ACTGGCAATG	CCGGGATAGA	CTGTAACATT
1990	2000	2010	2020	2030	2040
CTCACGCATA	AAATCCCCTT	TCATTTTCTA	ATGTAAATCT	ATTACCTTAT	TATTAATTCA
2050	2060	2070	2080	2090	2100
ATTTCGTCAT	AATTAATCCT	TTTTCTTATT	ACGCAAAATG	GCCCCGATTTA	AGCACACCCT
2110	2120	2130	2140	2150	2160
TTATTCCGTT	AATGCGCCAT	GACAGCCATG	ATAATTACTA	ATACTAGGAG	AAGTTAATAA

2170 2180 2190 2200 2210 2220
ATACGTAACC AACATGATTA ACAATTATTA GAGGTCATCG TTCAAAATGG TATGCGTTTT
 2230 2240 2250 2260 2270 2280
GACACATCCA CTATATATCC GTGTCGTTCT GTCCACTCCT GAATCCCAT CCAGAAATTC
 2290 2300 2310 2320 2330 2340
TCTAGCGATT CCAGAAGTTT CTCAGAGTCG GAAAGTTGAC CAGACATTAC GAAGTGGCAC
 2350 2360 2370 2380 2390 2400
AGATGGTCAT AACCTGAAGG AAGATCTGAT TGCTTAACTG CTTCAGTTAA GACCGAAGCG
 2410 2420 2430 2440 2450 2460
CTCGTCGTAT AACAGATGCG ATGATGCAGA CCAATCAACA TGGCACCTGC CATTGCTACC
 2470 2480 2490 2500 2510 2520
TGTACAGTCA AGGATGGTAG AAATGTTGTC GGTCCTTGCA CACGAATATT ACGCCATTTG
 2530 2540 2550 2560 2570 2580
CCTGCATATT CAAACAGCTC TTCTACGATA AGGGCACAAA TCGCATCGTG GAACGTTTGG
 2590 2600 2610 2620 2630 2640
GCTTCTACCG ATTTAGCAGT TTGATACACT TTCTCTAAGT ATCCACCTGA ATCATAAATC
 2650 2660 2670 2680 2690 2700
GGCAAAATAG AGAAAAATTG ACCATGTGTA AGCGGCCAAT CTGATTCCAC CTGAGATGCA
 2710 2720 2730 2740 2750 2760
TAATCTAGTA GAATCTCTTC GCTATCAAAA TTCACTTCCA CCTTCCACTC ACCGGTTGTC
 2770 2780 2790 2800 2810 2820
CATTCTATGGC TGAACTCTGC TTCTCTGT GACATGACAC ACATCATCTC AATATCCGAA
 2830 2840 2850 2860 2870 2880
TAGGGCCCAT CAGTCTGACG ACCAAGAGAG CCATAAACAC CAATAGCCTT AACATCATCC
 2890 2900 2910 2920 2930 2940
CCATATTTAT CCAATATTCG TTCTTAATT TCATGAACAA TCTTCATTCT TTCTTCTCTA
 2950 2960 2970 2980 2990 3000
GTCATTATTA TTGGTCCATT CACTATTCTC ATTCCCTTTT CAGATAATTT TAGATTGCT
 3010 3020 3030 3040 3050 3060
TTTCTAAATA AGAATATTTG GAGAGCACCG TTCTTATTCA GCTATTAATA ACTCGTCTTC
 3070 3080 3090 3100 3110 3120
CTAAGCATCC TTCAATCCTT TTAATAACAA TTATAGCATC TAATCTTCAA CAAAGTGGCC
 3130 3140 3150 3160 3170 3180
CGTTTGTTGA ACTACTCTTT AATAAAATAA TTTTTCCGTT CCCAATTCCA CATTGCAATA
 3190 3200 3210 3220 3230 3240
ATAGAAAATC CATCTTTCATC GGCTTTTTTCG TCATCATCTG TATGAATCAA ATCGCCTTCT

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      3250      3260      3270      3280      3290      3300
TCTGTGTCAT CAAGGTTTAA TTTTTTATGT ATTTCTTTTA ACAAACCACC ATAGGAGATT

      3310      3320      3330      3340      3350      3360
AACCTTTTAC GGTGTAAACC TTCTTCCAAA TCAGACAAAC GTTTCAAATT CTTTCTTCA

      3370      3380      3390      3400      3410      3420
TCATCGGTCA TAAATCCGT ATCCTTTACA GGATATTTTG CAGTTTCGTC AATTGCCGAT

      3430      3440      3450      3460      3470      3480
TGTATATCCG ATTTATATTT ATTTTTCGGT CGAATCATTT GAACTTTTAC ATTTGGATCA

      3490      3500      3510      3520      3530      3540
TAGTCTAATT TCATTGCCTT TTTCCAAAAT TGAATCCATT GTTTTTGATT CACGTAGTTT

      3550      3560      3570      3580      3590      3600
TCTGTATTCT TAAATAAGT TGGTTCACA CATACCAATA CATGCATGTG CTGATTATAA

      3610      3620      3630      3640      3650      3660
GAATTATCTT TATTATTTAT TGTCACTTCC GTTGCACGCA TAAACCAAC AAGATTTTAA

      3670      3680      3690      3700      3710      3720
TTAATTTTTT TATATTGCAT CATTTCGGCGA AATCCTTGAG CCATATCTGA CAAACTCTTA

      3730      3740      3750      3760      3770      3780
TTTAATTCCT CGCCATCATA AACATTTTTA ACTGTTAATG TGAGAAACAA CCAACGAAC

      3790      3800      3810      3820      3830      3840
GTTGGCTTTT GTTAAATAAC TTCAGCAACA ACCTTTTGTG ACTGAATGCC ATGTTTCATT

      3850      3860      3870      3880      3890      3900
GCTCTCCTCC AGTTGCACAT TGGACAAAGC CTGGATTTAC AAAACCACAC TCGATACAAC

      3910      3920      3930      3940      3950      3960
TTTCTTTTCG CTGTTTCACG ATTTTGTTTA TACTCTAATA TTTCAGCACA ATCTTTTACT

      3970      3980      3990      4000      4010      4020
CTTTCAGCCT TTTTAAATTC AAGAATATGC AGAAGTTCAA AGTAATCAAC ATTAGCGATT

      4030      4040      4050      4060      4070      4080
TTCTTTTCTC TCCATGGTCT CACTTTTCCA CTTTTTGTCT TGTCCACTAA AACCCCTTGAT

      4090      4100      4110      4120      4130      4140
TTTTTCATCTG AATAAATGCT ACTATTAGGA CACATAAATAT TAAAAGAAAC CCCCATCTAT

      4150      4160      4170      4180      4190      4200
TTAGTTATTT GTTTAGTCAC TTATAACTTT AACAGATGGG GTTTTTCTGT GCAACCAATT

      4210      4220      4230      4240      4250      4260
TTAAGGGTTT TCAATACTTT AAAACACATA CATACCAACA CTTCAACGCA CCTTTCAGCA

      4270      4280      4290      4300      4310      4320
ACTAAAATAA AAATGACGTT ATTTCTATAT GTATCAAGAT AAGAAAGAAC AAGTTCAAAA

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4330	4340	4350	4360	4370	4380
CCATCAAAAA	AAGACACCTT	TTCAGGTGCT	TTTTTTATTT	TATAAACTCA	TTCCCTGATC
4390	4400	4410	4420	4430	4440
TCGACTTCGT	TCTTTTTTTA	CCTCTCGGTT	ATGAGTTAGT	TCAAATTCGT	TCTTTTTAGG
4450	4460	4470	4480	4490	4500
TTCTAAATCG	TGTTTTTCTT	GGAATTGTGC	TGTTTTATCC	TTTACCTGT	CTACAAACCC
4510	4520	4530	4540		
CTTAAAAACG	TTTTTAAAGG	CTTTTAAGCC	GTCTGTACGT	TCCTTAAG	

A search in pUB110

AccI	4489
AhaIII(DraI)	41 62 499 1320 1381 3972
	4218 4513
AluI	508 1034 1420 1584 1605 1818
	2536 3040
ApaI	2823
AvaI	580
AvaII	2491 2953
BamHI	788
BanI	2442
BanII	2823
BbvI	245
BglII	2362
BsmI	1051
	3824
BspMI	1272 2445
BstXI	1879
DdeI	7 255 1288 1438 2301 2615
	2691 3061
EcoO109	2822
EcoRI	1
EcoRII(BstNI)	785 3870
FnuDII	1548
Fnu4HI	245 1744 2672
FokI	1650 2225 2876 3066
	2472
HaeII	1812 2397
HaeIII	2080 2674 2824 3117
HgiAI	1313
	3023
HhaI(CfoI,HinPI)	1549 1813 2114 2398
HincII(HindII)	2315 2788
HinfI	885 1436 1772 1796 1844 1875
	2261 2287 2305 2629 2683 2711
	3224 3452 3512 3527
HpaII(MspI)	1128 1791 1961 2752
HphI	1793 1889
	350 542 2749
MboI(Sau3AI,Dpn)	789 1359 1626 1662 2363 3476
	4377
MboII	1356 2360
	77 1882 2539 2716 2921 2932
	3056 3104 3193 3238 3355 3727
MnlI	762 856 1278 1287 1731 1832
	1853 2783 3323 3846 4401
	681 1110 1193 1519 2191
NciI	1961
NcoI	4032
NdeI	574 1073
NlaIII	156 202 216 340 376 676
	964 1019 1886 2118 2127 2173
	2439 2663 2765 2793 2912 3581

NlaIII	3585 3830 4033
NlaIV	788 2442 2823 3562
NsiI	673 2696 3582
PvuII	1033
RsaI	197 393 870 1757 1784 2462
	4535
Sau96I	2080 2491 2823 2824 2953 3117
ScaI	196
ScrFI	785 1961 3870
SfaNI	848 2563 3065 3096 3677
	2415 2423 2695
SnaBI	2162
SphI	1018
SspI	718 920 2505 2893 3013 3937
	4116
StyI	4032
TaqI	3450 3891 4381
XbaI	464
XhoII	788 2362
XmnI	2711 4307

these sequences did not occur at all

AatI	AhaII	
BalI		BclI
BglI		BspMII
BssHII	Bst6II	ClaI
CvnI	HgaI	EcoRV
	HindIII	HgiAI
KpnI	MluI	HpaI
MstII	NaeI	MstI
	NheI	NarI
NruI	Paer7I	NotI
	PstI	PpuMI
RsrII		PvuI
SacII(SstII)	SalI	SacI
SmaI(XmaI)	SpeI	SfiI
StuI		SstI
	Tth111I	
XmaIII		XhoI

The Nucleotide Sequence and Restriction Site Analysis of pE194 (Clockwise Relative to Fig. 2, Chapter 6, Section 3.2)

IV

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10	20	30	40	50	60
<u>GATCAGTACA</u>	AGAAAGATAC	TGTATTTCAT	AAACAGGAAC	TGCAAGAAGT	TAAGGATGAG
70	80	90	100	110	120
TTACAGAAGG	CAAATAAGCA	GTTACAGAGT	GGAATAGAGC	<u>ATATGAGGTC</u>	<u>TACGAAACCC</u>
130	140	150	160	170	180
TTTGATTATG	AAAATGAGCG	<u>TACAGGTTTG</u>	TTCTCTGGAC	<u>GTGAAGAGAC</u>	TGGTAGAAAG
190	200	210	220	230	240
ATATTAACTG	CTGATGAATT	TGAACGCCTG	CAAGAAACAA	<u>TCTCTTCTGC</u>	<u>AGAACGGATT</u>
250	260	270	280	290	300
GTTGATGATT	ACGAAAATAT	<u>TAAGAGCACA</u>	GACTATTACA	CAGAAAATCA	AGAATTAAAA
310	320	330	340	350	360
AAACGTAGAG	AGAGTTTGAA	AGAAGTAGTG	AATACATGGA	<u>AAGAGGGGTA</u>	TCACGAAAAA
370	380	390	400	410	420
AGTAAAGAGG	TTAATAAATT	AAAGCGAGAG	AATGATAGTT	TGAATGAGCA	GTTGAATGTA
430	440	450	460	470	480
TCAGAGAAAT	TTCAAGCTAG	<u>TACAGTGACT</u>	TTATATCGTG	<u>CTGCGAGGGC</u>	<u>GAATTTCCCT</u>
490	500	510	520	530	540
<u>GGGTTTGAGA</u>	AAGGGTTTAA	TAGGCTTAAA	GAGAAATTCT	TTAATGATTTC	CAAATTTGAG
550	560	570	580	590	600
CGTGTGGGAC	AGTTTATGGA	<u>TGTTGTACAG</u>	GATAATGTCC	<u>AGAAGGTCGA</u>	TAGAAAGCGT
610	620	630	640	650	660
GAGAAACAGC	<u>GTACAGACGA</u>	TTTAGAGATG	<u>TAGAGGTACT</u>	TTTATGCCGA	GAAAACTTTT
670	680	690	700	710	720
TGCGTGTGAC	AGTCCTTAAA	<u>ATATACTTAG</u>	AGCGTAAGCG	AAAGTAGTAG	CGACAGCTAT
730	740	750	760	770	780
TAACTTTCGG	TTGCAAAGCT	CTAGGATTTT	TAATGGACGC	<u>AGCGCATCAC</u>	ACGCAAAAAG
790	800	810	820	830	840
GAAATTGGAA	TAAATGCGAA	ATTTGAGATG	TTAATTAAAG	ACCTTTTGA	<u>GGTCTTTTTT</u>
850	860	870	880	890	900
<u>TCTTAGATTT</u>	TTGGGGTTAT	TTAGGGGAGA	AAACATAGGG	<u>GGGTACTACG</u>	<u>ACCTCCCCCC</u>
910	920	930	940	950	960
<u>TAGGTGTCCA</u>	TTGTCCATTG	TCCAAACAAA	TAAATAAATA	<u>TTGGGTTTTT</u>	AATGTTAAAA
970	980	990	1000	1010	1020
GGTTGTTTTT	TATGTTAAAG	TGAAAAAAAC	AGATGTTGGG	<u>AGGTACAGTG</u>	ATAGTTGTAG
1030	1040	1050	1060	1070	1080
ATAGAAAAGA	<u>AGAGAAAAAA</u>	GTTGCTGTTA	CTTTAAGACT	TACAACAGAA	<u>GAAAAATGAGA</u>

1090	1100	1110	1120	1130	1140
TATTAAATAG	<u>AATCAAAGAA</u>	AAATATA <u>ATA</u>	<u>TTAGCAAATC</u>	<u>AGATGCAACC</u>	<u>GGTATTCTAA</u>
1150	1160	1170	1180	1190	1200
TAAAAAATA	TGCAAAGGAG	<u>GAATACGGTG</u>	<u>CATTTTAAAC</u>	AAAAAAAGAT	AGACAGCACT
1210	1220	1230	1240	1250	1260
<u>GGCATGCTGC</u>	CTATCTATGA	CTAAATTTTG	TTAAGTGAT	TAGCACCGTT	ATTATATCAT
1270	1280	1290	1300	1310	1320
<u>GAGCGAAAAT</u>	GTAATAAAAG	AAACTGAAAA	CAAGAAAAAT	TCAAGAGGAC	GTAATTGGAC
1330	1340	1350	1360	1370	1380
ATTTGTTTTA	<u>TATCCAGAAT</u>	<u>CAGCAAAAGC</u>	CGAGTGGTTA	GAGTATTTAA	<u>AAGAGTTTCA</u>
1390	1400	1410	1420	1430	1440
CATTCAATTT	GTAGTGTCTC	CATTACATGA	TAGGGATACT	GATACAGAAG	GTAGGATGAA
1450	1460	1470	1480	1490	1500
AAAAGAGCAT	TATCATATTC	TAGTGATGTA	<u>TGAGGGTAAT</u>	AAATCTTATG	AACAGATAAA
1510	1520	1530	1540	1550	1560
AATAATTAAC	<u>AGAAGAATTG</u>	<u>AATGCGACTA</u>	TTCCGCAGAT	TGCAGGAAGT	GTGAAAGGTC
1570	1580	1590	1600	1610	1620
TTGTGAGATA	TATGCTTCAC	<u>ATGGACGATC</u>	CTAATAAATT	<u>TAAATATCAA</u>	<u>AAAGAAGATA</u>
1630	1640	1650	1660	1670	1680
TGATAGTTTA	TGGCGGTGTA	GATGTTGATG	AATTATTAAA	GAAAACAACA	ACAGATAGAT
1690	1700	1710	1720	1730	1740
ATAAATTAAT	TAAAGAAATG	ATTGAGTTTA	TTGATGAACA	<u>AGGAATCGTA</u>	GAATTTAAGA
1750	1760	1770	1780	1790	1800
GTTTAATGGA	TTATGCAATG	<u>AAGTTTAAAT</u>	TTGATGATTG	<u>GTTCCCGCTT</u>	TTATGTGATA
1810	1820	1830	1840	1850	1860
ACTCGGCGTA	TGTTATTCAA	GAATATATAA	AATCAAATCG	GTATAAATCT	GACCGATAGA
1870	1880	1890	1900	1910	1920
TTTTGAATTT	AGGTGTCACA	AGACACTCTT	TTTTCGCACC	AGCGAAAAC	GGTTTAAGCC
1930	1940	1950	1960	1970	1980
<u>GACTGCGCAA</u>	AAGACATAAT	<u>CGATTACAAA</u>	AAAATAGGCA	CACGAAAAAC	<u>AAGTTAAGGG</u>
1990	2000	2010	2020	2030	2040
<u>ATGCAGTTTA</u>	<u>TGCATCCCTT</u>	<u>AACTTACTTA</u>	TTAAATAATT	TATAGCTATT	GAAAAGAGAT
		End erm			
2050	2060	2070	2080	2090	2100
AAGAATTGTT	<u>CAAAGCTAAT</u>	<u>ATTGTTTAAA</u>	TCGTCAATTC	<u>CTGCATGTTT</u>	TAAGGAATTG
2110	2120	2130	2140	2150	2160
TTAAATTGAT	TTTTTGTA <u>AA</u>	<u>TATTTCTTGG</u>	<u>TATTCCTTGT</u>	<u>TAACCCATTT</u>	CATAACGAAA

2170	2180	2190	2200	2210	2220
TAATTATACT	TTTGTTTATC	TTTGTGTGAT	ATTCTTGATT	TTTTTCTACT	TAATCTGATA
2230	2240	2250	2260	2270	2280
AGTGAGCTAT	TCACCTTAGG	TTTAGGATGA	<u>AAATATTCTC</u>	<u>TTGGAACCAT</u>	ACTTAATATA
2290	2300	2310	2320	2330	2340
GAAATATCAA	CTTCTGCCAT	TAAAAGTAAT	GCCAATGAGC	GTTTTGTATT	TAATAATCTT
2350	2360	2370	2380	2390	2400
TTAGCAAACC	CGTATTCCAC	GATTAAATAA	ATCTCATTAG	<u>CTATACTATC</u>	AAAAACAATT
2410	2420	2430	2440	2450	2460
TTGCGTATTA	<u>TATCCGTACT</u>	TATGTTATAA	GGTATATTAC	CATATATTTT	ATAGGATTGG
2470	2480	2490	2500	2510	2520
TTTTTAGGAA	<u>ATTTAAACTG</u>	CAATATATCC	<u>TTGTTTAAAA</u>	<u>CTTGGAATTT</u>	<u>ATCGTGATCA</u>
2530	2540	2550	2560	2570	2580
ACAAGTTTAT	TTTCTGTAGT	TTTGCATAAT	TTATGGTCTA	TTTCAATGGC	AGTTACGAAA
2590	2600	2610	2620	2630	2640
TTACACCTCT	TTACTAATTC	AAGGGTAAAA	<u>TGGCCTTTTC</u>	<u>CTGAGCCGAT</u>	TTCAAAGATA
2650	2660	2670	2680	2690	2700
TTATCATGTT	CATTTAATCT	TATATTGTGC	ATTATTTTAT	CTATATTATG	TTTTGAAGTA
2710	2720	2730	2740	2750	2760
ATAAAGTTTT	GACTGTGTTT	TATATTTTTC	<u>TCGTTTCATTA</u>	<u>TAACCCCTCTT</u>	TAATTTGGTT
			Start erm		
2770	2780	2790	2800	2810	2820
ATATGAATTT	TGCTTATTAA	<u>CGATTTCATTA</u>	TAACCACTTA	TTTTTTGTTT	GGTTGATAAT
2830	2840	2850	2860	2870	2880
GAACTGTGCT	GATTACAAAA	ATACTAAAAA	TGCCCATATT	<u>TTTTCCCTCCT</u>	TATAAAATTA
2890	2900	2910	2920	2930	2940
GTATAATTAT	<u>AGCACGAGCT</u>	<u>CTGATAAATA</u>	<u>TGAACATGAT</u>	<u>GAGTGATCGT</u>	TAAATTTATA
2950	2960	2970	2980	2990	3000
CTGCAATCGG	<u>ATGCGATTAT</u>	TGAATAAAAG	ATATGAGAGA	TTTATCTAAT	TTCTTTTTTC
3010	3020	3030	3040	3050	3060
TTGTAAAAAA	AGAAAGTTCT	TAAAGGTTTT	ATAGTTTTTG	<u>TCGTAGAGCA</u>	<u>CACGGTTTAA</u>
3070	3080	3090	3100	3110	3120
CGACTTAATT	ACGAAGTAAA	TAAGTCTAGT	GTGTTAGACT	TTATGAAATC	TATATACGTT
3130	3140	3150	3160	3170	3180
TATATATATT	TATTATCCGG	<u>AGGTGTAGCA</u>	<u>TGTCTCATTC</u>	<u>AATTTTGAGG</u>	GTTGCCAGAG
3190	3200	3210	3220	3230	3240
<u>TTAAAGGATC</u>	AAGTAATACA	AACGGGATAC	AAAGACATAA	TCAAAGAGAG	AATAAAAACT

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      3250      3260      3270      3280      3290      3300
ATAATAATAA AGACATAAAT CATGAGGAAA CATATAAAAA TTATGATTTG ATTAACGCAC

      3310      3320      3330      3340      3350      3360
AAAATATAAA GTATAAAGAT AAAATTGATG AAACGATTGA TGAGAATTAT TCAGGGAAAC

      3370      3380      3390      3400      3410      3420
GTAAAATTCG GTCAGATGCA ATTTCGACATG TGGACGGACT GGTTACAAGT GATAAAGATT

      3430      3440      3450      3460      3470      3480
TCTTTGATGA TTTAAGCGGA GAAGAAATAG AACGATTTTT TAAAGATAGC TTGGAGTTTC

      3490      3500      3510      3520      3530      3540
TAGAAAATGA ATACGGTAAG GAAAATATGC TGTATGCGAC TGTCCATCTG GATGAAAGAG

      3550      3560      3570      3580      3590      3600
TCCCACATAT GCACTTTGGT TTTGTCCCTT TAACAGAGGA CGGGAGATTG TCTGCAAAAG

      3610      3620      3630      3640      3650      3660
AACAGTTAGG CAACAAGAAA GACTTTACTC AATTACAAGA TAGATTTAAT GAGTATGTGA

      3670      3680      3690      3700      3710      3720
ATGAGAAAGG TTATGAACCTT GAAAGAGGCA CGTCCAAAGA GGTTACAGAA CGAGAACATA

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AAGCGATG

A search in pE194

AccI	108
AhaIII(DraI)	1174 1366 1599 1764 2065 2472
	2494 3459
AluI	435 715 737 2024 2054 2225
	2379 2897 3468
BanII	2896
BbvI	460 1206
	759
BclI	2515
BsmI	1520
BspMII	3136
ClaI	1939
DdeI	686 842 2621
EcoRII(BstNI)	478
Fnu4HI	460 759 1206
FokI	1993
	54 558 1434 1979 2245 2949
	3530
HaeIII	2612
HgaI	756
HqiAI	264 3046
	2896
HhaI(CfoI,HinPI	762 1925
HincII(HindII)	2139
HinfI	526 1090 1337 1723 1942 2782
	3538
HpaI	2139
HpaII(MspI)	1129 3137
MboI(Sau3AI,Dpn	1 1587 2516 2925 3187
MboII	163 1029 1068 1512 1614 3441
	223
MnlI	892 2586 2745 2865
	105 343 367 465 633 829
	1000 1158 1305 1472 3140 3167
	3264 3576 3685 3699
MstI	1924
NdeI	100 3546
NlaIII	335 1203 1258 1406 1580 2084
	2645 2915 3149 3261 3387
NlaIV	1780 2263
NsiI	1990
PstI	227
RsaI	6 140 440 565 611 636
	883 1003 2416
SacI	2896
ScrFI	478
SfaNI	764 1992
	1122 1980 2950 3375
SphI	1202
SspI	256 937 1107 2058 2119 2252
SstI	2896
StyI	899

TaqI	587 1940 3383
XbaI	3479
XmnI	2249

These sequences did not occur at all

AatI	AhaII	ApaI
AvaI	AvaII	
BalI	BamHI	BanI
BglI	BglII	
BspMI		BssHII
BstEII	BstXI	CvnI
EcoO109	EcoRI	
EcoRV	FnuDII	HaeII
HindIII	HphI	
KpnI	MluI	MstII
NaeI	NarI	NciI
	NcoI	NheI
NotI	NruI	PaeR71
PpuMI		PvuI
PvuII	RsrII	
SacII(SstII)	SalI	Sau96I
ScaI	SfiI	SmaI(XmaI)
SnaBI	SpeI	StuI
Tth111I	XhoI	XhoII
XmaIII		

The Nucleotide Sequence and
Restriction Site Analysis of pC194
(Clockwise Relative to Fig. 3,
Chapter 6, Section 3.3)

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10	20	30	40	50	60
<u>GATCAATTC</u>	TTTAAAGTAA	TCTAAATCCC	CATTTTTTTAA	TTTCTTTTTTA	<u>GCCTCTTTAA</u>
70	80	90	100	110	120
<u>ATAATCCTGA</u>	ATAAACTAAT	ACCTGTTTAC	CTTTAAGTGA	TTTATAAAAT	<u>GCATCAAAGA</u>
130	140	150	160	170	180
CTTTTTGATT	TATTAAATAA	TCACTATCTT	TACCAGAATA	<u>CTTAGCCATT</u>	TCATATAATT
190	200	210	220	230	240
CTTTATTATT	ATTTTGTCTT	ATTTTTTGAA	CTTGAACCTG	TGTTATTTCT	GAAATGCCCG
250	260	270	280	290	300
TTACATCACG	CCATAAATCT	AACCATTCTT	GTTGGCTAAT	ATAATATCTT	TTATCTGTGA
310	320	330	340	350	360
AATACGATTT	ATTTACTGCA	ATTAACACAT	<u>GAAAAAGAGG</u>	ATTATAATCA	TCTCTTTTTT
370	380	390	400	410	420
TATTATATGT	AATCTCTAAC	TTACGAACAT	ATCCCTTTAT	AACACTACCT	ACTTTTTTTC
430	440	450	460	470	480
TCTTTATAAG	TTTTCTAAAA	GAATTATTAT	AACGTTTTAT	TTCATTTTTCT	AATTCATCAC
490	500	510	520	530	540
TCATTACATT	AGGTGTAGTC	AAAGTTAAAA	AGATAAACTC	CTTTTCTCTCT	<u>TGCTGCTTAA</u>
550	560	570	580	590	600
TATATTGCAT	<u>CATCAAAGAT</u>	AAACCCAAATG	<u>CATCTTTTCT</u>	<u>AGCTTTTCTC</u>	CAAGCACAGA
610	620	630	640	650	660
CAGGACAAAA	<u>TCGATTTTTTA</u>	CAAGAATTAG	<u>CTTTATATAA</u>	TTTCTGTTTT	TCTAAAGTTT
670	680	690	700	710	720
TATCAGCTAC	AAAAGACAGA	AATGTATTGC	<u>AATCTTCAAC</u>	TAAATCCATT	<u>TGATTCTCTC</u>
730	740	750	760	770	780
CAATATGACG	TTTAATAAAT	TTCTGAAATA	CTTGATTTCT	TTGTTTTTTC	<u>TCAGTATACT</u>
790	800	810	820	830	840
TTTCCATGTT	ATAACACATA	AAAACAACCTT	<u>AGTTTTTCA</u>	AACTATGACA	ATAAAAAAAG
850	860	870	880	890	900
TTGCTTTTTT	CCCTTTCTAT	GTATGTTTTT	<u>TACTAGTCAT</u>	<u>TTAAAACGAT</u>	ACATTAATAG
910	920	930	940	950	960
<u>GTACGAAAAA</u>	GCAACTTTTT	<u>TTGCGCTTAA</u>	AACCAGTCAT	ACCAATAACT	TAAGGGTAAC
970	980	990	1000	1010	1020
<u>TAGCCTCGCC</u>	<u>GGCAATAGTT</u>	ACCCTTATTA	TCAAGATAAG	AAAGAAAAGG	ATTTTTTCGCT
1030	1040	1050	1060	1070	1080
ACGCTCAAAT	<u>CCTTTAAAAA</u>	AACACAAAAG	ACCACATTTT	TTAATGTGGT	<u>CTTTATTCTT</u>

1090	1100	1110	1120	1130	1140
<u>CAACTAAAGC</u>	ACCCATTAGT	TCAACAAACG	AAAATTGGAT	AAAGTGGGAT	ATTTTTTAAA
1150	1160	1170	1180	1190	1200
TATATATTTA	TGTTACAGTA	<u>ATATTGACTT</u>	<u>TTAAAAAAGG</u>	ATTGATTCTA	ATGAAGAAAG
1210	1220	1230	1240	1250	1260
CAGACAAGTA	<u>AGCCTCCTAA</u>	ATTCACITTTA	GATAAAAAATT	TAGGAGGCAT	ATCAAATGAA
1270	1280	1290	1300	1310	1320
CTTTAATAAA	ATTGATTTAG	ACAATTGGAA	<u>GAGAAAAGAG</u>	ATATTTAATC	ATTATTTGAA
1330	1340	1350	1360	1370	1380
CCAACAAACG	ACTTTTAGTA	TAACCACAGA	AATTGATATT	AGTGTTTTAT	ACCGAAACAT
1390	1400	1410	1420	1430	1440
AAAACAAGAA	GGATATAAAT	TTTACCCTGC	ATTTATTTTC	<u>TTAGTGACAA</u>	<u>GGGTGATAAA</u>
1450	1460	1470	1480	1490	1500
CTCAAATACA	<u>GCTTTTAGAA</u>	CTGGTTACAA	TAGCGACGGA	GAGTTAGGTT	ATTGGGATAA
1510	1520	1530	1540	1550	1560
GTTAGAGCCA	CTTTATACAA	TTTTTGATGG	TGTATCTAAA	ACATTCTCTG	GTATTTGGAC
1570	1580	1590	1600	1610	1620
<u>TCCTGTAAAG</u>	<u>AATGACTTCA</u>	AAGAGTTTTA	TGATTTATAC	CTTTCTGATG	TAGAGAAATA
1630	1640	1650	1660	1670	1680
TAATGGTTCG	GGGAAATTGT	TTCCCAAAAC	ACCTATACCT	GAAAATGCTT	TTTCTCTTTC
1690	1700	1710	1720	1730	1740
TATTATTCCA	<u>TGGACTTCAT</u>	TTACTGGGTT	TAACTTAAAT	ATCAATAATA	ATAGTAATTA
1750	1760	1770	1780	1790	1800
CCTTCTACCC	ATTATTACAG	CAGGAAAATT	CATTAATAAA	GGTAATTCAA	TATATTTACC
1810	1820	1830	1840	1850	1860
GCTATCTTTA	CAGGTACATC	ATTCTGTTTG	TGATGGTTAT	<u>CATGCAGGAT</u>	TGTTTATGAA
1870	1880	1890	1900	1910	1920
CTCTATTTCAG	GAATTGTCAG	ATAGGCCTAA	TGACTGGCTT	<u>TTATAATATG</u>	AGATAATGCC
1930	1940	1950	1960	1970	1980
GACTGTACTT	TTTACAGTCG	GTTTTCTAAT	GTCACTAACC	<u>TGCCCCGTTA</u>	GTTGAAGAAG
1990	2000	2010	2020	2030	2040
GTTTTTATAT	TACAGCTCCA	<u>GATCCATATC</u>	CTTCTTTTTC	TGAACCGACT	TCTCCTTTTT
2050	2060	2070	2080	2090	2100
CGCTTCTTTA	TTCCAATTGC	TTTATTGACG	TTGAGCCTCG	<u>GAACCCCTAA</u>	CAATCCCAAA
2110	2120	2130	2140	2150	2160
ACTTGTCGAA	TGGTCGGCTT	AATAGCTCAC	GCTATGCCGA	CATTCGTCGT	CAAGTTTAGT

2170	2180	2190	2200	2210	2220
TAAGGGT <u>TCT</u>	<u>TCT</u> CAACGCA	CAATAAA <u>TTT</u>	TCTCGGCATA	AATGCGTGGT	CTAATTTTTTA
2230	2240	2250	2260	2270	2280
TTTTTAATAA	CCTTGATAGC	AAAAAATGCC	ATTCCAATAC	AAAACCACAT	ACCTATA <u>AATC</u>
2290	2300	2310	2320	2330	2340
<u>GATA</u> ACCACA	TAACAGTCAT	AAAACCACTC	CTTTTTAACA	AACTTTATCA	CAAGAA <u>AATAT</u>
2350	2360	2370	2380	2390	2400
<u>TTAAATTTTA</u>	<u>AATG</u> CCTTTA	TTTTGAATTT	TAAGGGGCAT	<u>TTTAA</u> AGATT	TAGGGGTAAA
2410	2420	2430	2440	2450	2460
TCATATAGTT	TTATGCCTAA	AAACCTACAG	<u>AAGCTTTTAA</u>	<u>AAAG</u> CAAATA	TGAGCCAAAT
2470	2480	2490	2500	2510	2520
AAATATATTC	TAATTCTACA	AACAAAAATT	TGAGCAAATT	CAGT <u>GTCTGAT</u>	TTTTTAAGAC
2530	2540	2550	2560	2570	2580
ACTGCCCAGT	<u>TACATG</u> CAAA	TTAAAATTTT	<u>CATGAT</u> TTTT	TATAGTTCCT	AACAGGGTTA
2590	2600	2610	2620	2630	2640
AAATTTGTAT	AACGAAAGTA	TAATGTTTAT	ATAACGTTAG	TATAATAAAG	CATTTTAACA
2650	2660	2670	2680	2690	2700
TTATACTTTT	GATAATCGTT	TATCGTCGTC	ATCACAATAA	<u>CTTTTAA</u> AAT	ACTCGTGCAT
2710	2720	2730	2740	2750	2760
AATTCACGCT	<u>GACCTCC</u> CAA	TAAC <u>TACATG</u>	GTGTTATCGG	<u>GAGGT</u> CAGCT	<u>GTTAG</u> CACTT
2770	2780	2790	2800	2810	2820
ATATTTTGTT	ATT <u>GTCTCTC</u>	<u>CTCGA</u> TTTCG	TCTATCATTT	TGTGATTAAAT	TTCTCTTTTT
2830	2840	2850	2860	2870	2880
ICTTGTTCTG	TTAAGTCATA	AAGT <u>CTACTA</u>	<u>GCTAA</u> ATACT	CTTTTGTGTT	CCAAATATAA
2890	2900				
AAAATTTGAT	AGATATATTA	CGGTTG			

A search in pC194

AccI	774
AhaIII(DraI)	56 880 1033 1134 1170 2340
	2347 2381 2436 2683
AluI	581 629 665 1450 1994 2124
	2432 2747 2850
BbvI	532
BglI	963
BspMI	1958
ClaI	610 2278
DdeI	161 770 808 1420
Fnu4HI	532
HaeIII	1884
HhaI(CfoI,HinPI	923
HindIII	2431
HinfI	712 1184 1558
HpaII(MspI)	969
HphI	1432
MboI(Sau3AI,Dpn	1 2001
MboII	1193 1288 1974
	693 1077 2168 2776
MnlI	52 964 1213 2076 2713 2780
	337 1244 2741
NaeI	968
NcoI	1688
NlaIII	328 785 1689 1841 2533 2551
	2727
NlaIV	2080
NsiI	109 568
PvuII	2746
RsaI	901 1814 1925
SfaNI	111 547 570
SpeI	872
SspI	1160 2336
StuI	1883
StyI	1688
TaqI	611 2106 2279 2506 2782
XhoII	2000
XmnI	1570

These sequences did not occur at all

AatI	AhaII	ApaI
AvaI	AvaII	
BalI	BamHI	BanI
BanII		BclI
BglII	BsmI	
	BspMII	BssHII
BstEII	BstXI	CvnI
EcoO109	EcoRI	EcoRII(BstNI)
	EcoRV	FnuDII
FokI		HaeII

These sequences did not occur at all

HgaI		HgiAI
HincII(HindII)	HpaI	MstI
KpnI	MluI	NciI
MstII	NarI	NheI
	NdeI	PaeR71
NotI	NruI	PstI
PpuMI		
PvuI	RsrII	
SacI	SacII(SstII)	SalI
Sau96I	ScaI	ScrFI
	SfiI	SmaI(XmaI)
SnaBI	SphI	SstI
Tth111I	XbaI	XhoI
XmaIII		

Plasmid DNA Isolation

VI

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A log phase or an overnight culture of plasmid-containing *B. subtilis* is centrifuged and the pellet from 5 ml of the culture is resuspended in 200 μ l of a lysozyme solution (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0, plus 5 mg/ml of lysozyme added just prior to use). The amount of culture taken through the procedure can be scaled up by proportional increases in the volumes of the reagents. Cells are incubated with the lysozyme for 20 min at 37°C and 400 μ l of 0.2 M NaCl + 1% sodium dodecyl sulfate is added. After mixing on a Vortex, the solution becomes translucent and is placed on ice for 5 min. Three hundred microliters of 5 M potassium acetate, adjusted to pH 4.8 with glacial acetic acid, is added. After gentle mixing, a DNA clot forms. Place in dry ice-ethanol for 2 min, then centrifuge at room temperature for 20 min in a high-speed microfuge. Mix each 750 μ l of supernatant recovered with 450 μ l of isopropanol, place in dry ice-ethanol for 5 min, and centrifuge 5 min. Decant supernatant and wash pellet with cold 70% ethanol. Dry pellet under vacuum, add 100 μ l of Tris-EDTA buffer, and resuspend pellet by use of a Vortex. RNase (1 μ l of a 2 mg/ml solution) is added. If the DNA is to be used in restriction digests and ligations, we generally extract the DNA once with an equal volume of phenol and precipitate the DNA with cold 70% ethanol. The mixture is centrifuged for 5 min, washed a second time with 70% ethanol, and the final pellet is resuspended in 100 μ l of TE buffer. Although the plasmid DNA is enriched at this step and can be used for many studies, an additional purification step is achieved by centrifugation through an ethidium bromide CsCl gradient (see Lovett and Keggin, 1979, Chapter 4).

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Determination of Plasmid Copy Number VII

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Bacillus subtilis 168 contains no plasmids. Hence, centrifugation of total DNA from this strain in a CsCl-ethidium bromide gradient reveals only a single peak consisting of linear fragments of the chromosome. Plasmids are maintained in bacteria predominantly as covalently closed (supercoiled) DNA circles. Thus, CsCl-ethidium bromide centrifugation of the DNA from *B. subtilis* harboring pUB110 reveals two peaks. The major peak ($\rho = \sim 1.55$) consists of linear fragments of the chromosome plus open-circular and linear plasmid forms. The minor peak ($\rho = \sim 1.58$), of greater density than the chromosome peak, contains exclusively supercoiled (plasmid) DNA. Since the molecular weights of the *B. subtilis* chromosome ($\sim 2.5 \times 10^9$ Da) and pUB110 ($\sim 3 \times 10^6$ Da) are known, the number of pUB110 molecules per chromosome can be determined by calculating the percentage of the total DNA which is in the supercoiled configuration.

B. subtilis (pUB110) is grown in 10 ml of Spizizen minimal medium containing 250 $\mu\text{g/ml}$ of deoxyadenosine and 10 $\mu\text{Ci/ml}$ of [^3H]-thymidine. The total cellular DNA is assumed to become uniformly labeled. Cells are harvested during log phase growth. DNA can be extracted as described elsewhere (see Lovett, 1973, Chapter 6) or by a modification of the procedure described above in which the step separating bulk DNA from plasmid DNA is omitted. The DNA is mixed with ethidium bromide and CsCl to achieve 200 $\mu\text{g/ml}$ of the dye and an average buoyant density of 1.55. Centrifugation in a Ti50 rotor for 36 hr resolves supercoiled DNA from linear fragments and open circles. Gradients are fractionated into 30–40 fractions and each is precipitated and the precipitate dried onto a filter. The filters are counted for radioactivity and the percentage of the total recovered radioactivity which is in the peak of supercoiled DNA provides an estimate of the amount of plasmid DNA in cells. In our experience, at least 90% of the pUB110 molecules recovered from *B. subtilis* bands in the supercoiled satellite DNA peak. This was determined by hybridizing radioactively labeled pUB110 to unlabeled DNA recovered from the

two peaks (peaks were detected by fluorescence of the ethidium bromide under ultraviolet irradiation). Very large plasmids are more prone to nicking during the DNA isolation step. Thus, this method provides a lower estimate for plasmid copy number, which is reasonably accurate for small plasmids but possibly less accurate for relatively large plasmids (see Lovett and Bramucci, 1975, Chapter 6).

Analysis of Plasmid-Specified Proteins in Minicells

VIII

PAUL S. LOVETT and
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Bacillus subtilis strain CU403 (*thyA thyB metB divIVB1*) produces minicells during growth (see Reeve *et al.*, 1973, Chapter 6). When cells are grown in either a rich medium (e.g., Penassay broth; Difco) or an appropriately supplemented minimal medium (see Spizizen, 1958, Chapter 6), minicells accumulate in the culture and are easily seen by microscopic examination. To isolate these minicells, cultures are grown to late log phase and the cells are concentrated 100-fold, and then centrifuged through 5–30% sucrose gradients in 33-ml tubes (see Shivakumar *et al.*, 1979, Chapter 6) using an SW27 rotor at 5000 rpm for 20 min. The visible minicell band can be removed with a Pasteur pipette, the cells washed with cold minimal medium (10000 rpm) and recentrifuged through a second 5–30% sucrose gradient. The resulting minicells are washed with cold Spizizen minimal medium and stored in the same medium containing 10% glycerol. Frozen stocks reportedly can be stored at -70°C for 3 months with no loss of amino acid incorporation activity (see Shivakumar *et al.*, 1979, Chapter 6). Quantitation of minicells is performed by counting in a Petroff–Hauser chamber.

When plasmids are inserted into CU403, the recovered minicells contain the introduced plasmid. The higher the copy number of small plasmids (e.g., pUB110, pC194, or pE194), the higher the number of plasmids copies in the purified minicells.

In order to analyze plasmid proteins in purified minicells, plasmid-containing and plasmid-free minicells ($\sim 10^9/100\ \mu\text{l}$) are incubated at 32°C for 20 min and a radioactive amino acid is then added for 30–90 min, such as 40 $\mu\text{Ci/ml}$ of [^{35}S]-methionine. The cells are then diluted 10-fold in cold lysis medium (0.1 M EDTA, 0.05 M NaCl, 2 mM PMSF, 100 $\mu\text{g/ml}$ methionine, pH 6.9), pelleted, washed once with lysis medium, and resuspended in 1 ml of lysis medium. Fifty micrograms of lysozyme is added and the cells are held at 37°C for 15 min. Twenty-five microliters of cracking buffer is added (0.25% SDS, 1% mercaptoethanol, 0.05 M Tris-HCl pH 6.8, 0.002 M EDTA, and 10% glycerol). Samples are placed in a boiling water bath

for 3 min and electrophoresed through 10, 12.5, or 15% acrylamide gels. Gels are dried and exposed to X-ray film to detect protein bands.

Minicells lacking plasmid DNA show virtually no incorporation of radioactivity when incubated with [^{35}S]-methionine whereas plasmid-containing minicells cause incorporation of label into discrete protein bands. The number and the mobility of the bands reflects the specific plasmid in the minicells (see Shivakumar *et al.*, 1979, Chapter 6).

Single-Colony Lysis Gel Electrophoresis

IX

PAUL S. LOVETT and
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Plasmids present in individual colonies of *B. subtilis* can be detected by a scaled-down rapid lysis method. Individual plasmid-containing colonies of *B. subtilis* are transferred by toothpick to 25 μ l of lysozyme buffer (30 mM Tris, 50 mM EDTA, 50 mM NaCl, 25% sucrose, pH 8.0 with KOH; see Marrero and Lovett, 1980 and Lovett and Keggins, 1979, Chapter 6) containing 200 μ g/ml of lysozyme. The cells are incubated for 15 min at 37°C and a 100- μ l volume of SDS buffer is added (2 ml of 10% SDS, 2 ml of 500 mM disodium EDTA, pH 8.0 plus 12 ml TES buffer; see Lovett and Keggins, 1979, Chapter 6). Incubation is continued for 15 min at 37°C. A 40- μ l volume of 5 M NaCl is added and after vigorous agitation the tube is held in ice for 30 min. Tubes are then centrifuged in a microfuge and 50 μ l of the resulting supernatant fraction is electrophoresed through a 1% agarose gel.

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Updated Linkage Map of *B. Subtilis*

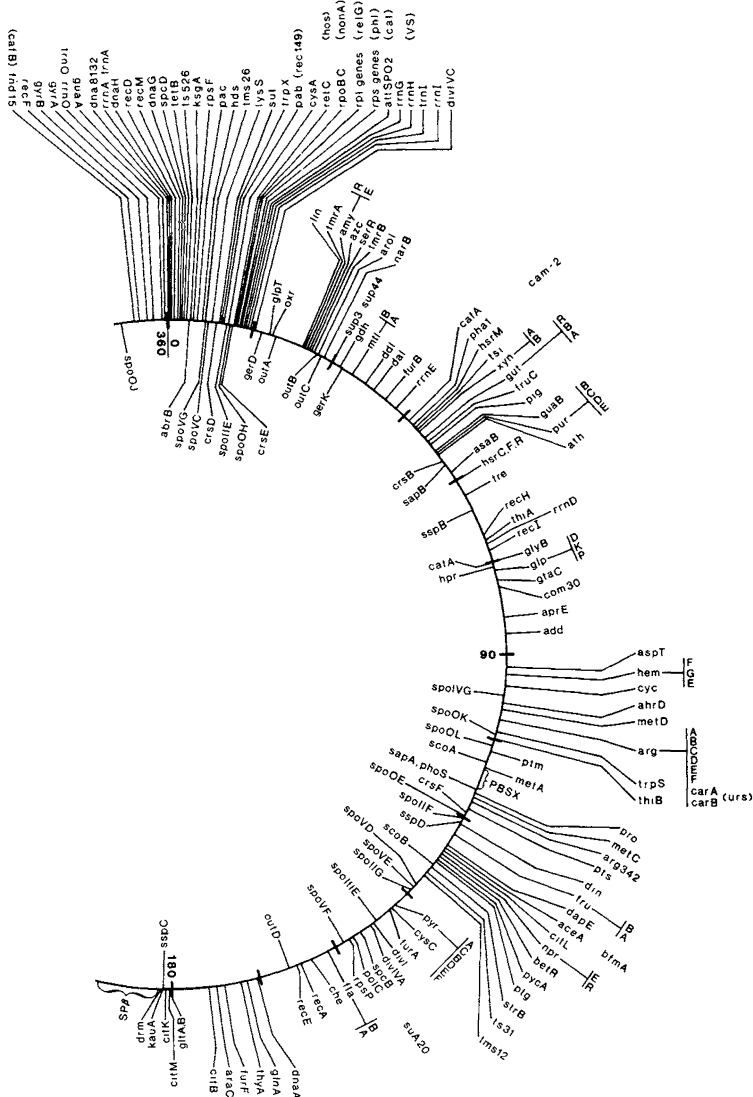
X

PATRICK J. PIGGOT and JAMES A. HOCH

Figure 1 gives the detailed genetic map of the *Bacillus subtilis* chromosome constructed according to data from the literature. Table I gives the detailed description of each locus or allele and references to the pertinent literature for each. It should be noted that these references are not exhaustive in the case of each locus, but rather were chosen for their particular information content with regard to this locus. It is important to emphasize that the order of the markers presented in this map is not cast in stone. Some areas of the genome are known quite accurately and therefore the order of the markers is well understood. This knowledge can extend to the level of the restriction map or even to that of the nucleotide sequence. The order of the markers within other regions may be substantially wrong as shown. This uncertainty results from the fact that it is precarious practice to order markers based on recombination distances in two factor crosses. In most of the chromosome we have had to order by this method because there were no available data from three factor genetic analyses. Thus some of the value of the information presented in this map is that it allows one to determine what loci have been mapped in the general area of a region of interest. It is difficult to know how accurate the map will be for any given region and the only solution for the curious is to contact the investigators who have done substantial mapping in the region of the chromosome of interest or, if all else fails, to read the original literature.

Figure 2 shows genetic maps of the region of the chromosome encoding the major cluster of ribosomal protein genes. The genetics of this region of the chromosome is particularly complex. We show here the available data in the literature with regard to the ordering of ribosomal genes in this region.

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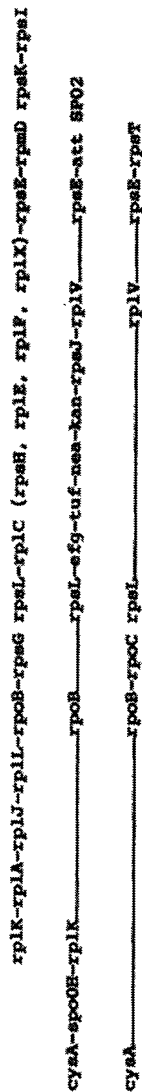


Figure 2. Partial maps of the principal cluster of ribosomal protein genes. The maps, from top to bottom, were derived from Refs. 51-55, 274, and 221, respectively. Other ribosomal protein genes located in this region include *rpsD*; *rplB* and *rplQ* (274); *rpsC*, *rpsQ*, and *rpsS* along with genes coding for BL4, BL14, BL22, and BL25 (213,221). Genes for particular proteins are given the name of the *Escherichia coli* homologue if known (51,117).

Table I. Genetic Markers of *B. subtilis*

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>abrA</i>	Antibiotic resistance	325	Partial suppressor of stage 0 phenotypes, may be same as <i>rev-4</i>	296
<i>abrB</i>	Antibiotic resistance	3	Partial suppressor of stage 0 phenotypes, ribosome alterations have been observed in these mutants; same locus as <i>cpsX</i> and probably <i>absA</i> , <i>absB</i> , and <i>tolA</i>	95, 297
<i>abrC</i>	Antibiotic resistance		Weak intragenic suppressors of <i>spo0A</i>	296
<i>absA</i>	Antibiotic resistance		Partial suppressor of stage 0 phenotypes, see <i>abrB</i>	138
<i>absB</i>	Antibiotic resistance		Partial suppressor of stage 0 phenotypes, see <i>abrB</i>	138
<i>aceA</i>	Acetate	126	Pyruvate dehydrogenase defect, defective in E1 (pyruvate decarboxylase) component of pyruvate dehydrogenase	26, 125
<i>acfA</i>	Acriflavin	230	Resistant to acriflavin, also to ethidium bromide and distamycin; sensitive to streptomycin	15, 134, 268
<i>acfB</i>	Acriflavin	215	Same as <i>acfA</i>	268
<i>add</i>	ATP ^c -dependent deoxyribonuclease	85	Gene for ATP-dependent deoxyribonuclease	B. Sedgwick and C. Anagnostopoulos (unpublished data)
<i>adeC</i>	Adenine	NM	Adenine deaminase	64
<i>adeF</i>	Adenine	NM	Adenine phosphoribosyltransferase	64
<i>aecA</i>	Aminoethylcysteine	250	Aminoethylcysteine resistance, regulation of aspartokinase II	179
<i>aecB</i>	Aminoethylcysteine	290	Aminoethylcysteine resistance, structural gene for aspartokinase II	179
<i>ahrA</i>	Arginine hydroxamate	342	Arginine hydroxamate resistance, linked to <i>cysA</i>	104, 201
<i>ahrB</i>	Arginine hydroximate	328	Arginine hydroxamate resistance	165, 201
<i>ahrC</i>	Arginine hydroximate	215	Arginine hydroxamate resistance	201
<i>ahrD</i>	Arginine hydroximate	99	Arginine hydroxamate resistance	17
<i>ala</i>	Alanine	281	Alanine auxotrophy	179
<i>ald</i>	Alanine	280	L-Alanine dehydrogenase	73, 165, 295
<i>alsA</i>	Acetoin	265	Acetolactate synthase	329
<i>alsR</i>	Acetoin	320	Constitutive acetolactate synthase	329

(continued)

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>amm</i>	Ammonia	250	Glutamate requirement	60
<i>amt</i>	Aminotyrosine		3-Aminotyrosine resistance; part of or very close to <i>tyrA</i> locus	230
<i>amyB</i>	Amylase		Control of amylase synthesis; probably identical to <i>sacQ</i> and <i>pap</i> ; see <i>sacQ</i>	261, 283
<i>amyE</i>	Amylase	25	Amylase structural gene, also called <i>amyA</i>	327
<i>amyR</i>	Amylase	25	Control of amylase synthesis, also called <i>amyH</i>	323, 327
<i>aprE</i>	Alkaline protease	91	Structural gene for subtilisin E, map order (<i>hpr glyB</i>)- <i>aprE</i> - <i>metD</i> (formerly <i>sprE</i>)	281, 321
<i>araA</i>	Arabinose	294	Arabinose utilization	221
<i>araB</i>	Arabinose	256	Arabinose utilization	221
<i>araC</i>	Arabinose	172	Arabinose utilization	221
<i>argA</i>	Arginine	100	Arginine requirement, identified by complementation of corresponding locus in <i>E. coli</i>	202
<i>argB</i>	Arginine	100	Same as <i>argA</i>	202
<i>argC</i>	Arginine	100	Same as <i>argA</i>	202
<i>argD</i>	Arginine	100	Same as <i>argA</i>	202
<i>argE</i>	Arginine	100	Same as <i>argA</i>	202
<i>argF</i>	Arginine	100	Same as <i>argA</i>	202
<i>argG</i>	Arginine	260	Same as <i>argA</i> , formerly <i>argA</i>	202
<i>argH</i>	Arginine	260	Same as <i>argG</i>	202
<i>arg342</i>	Arginine	115	Arginine-ornithine or citrulline requirement	117
<i>aroA</i>	Aromatic	265	3-Deoxy-D-arabinoheptalonic-7-phosphate synthase	119, 205
<i>aroB</i>	Aromatic	210	Dehydroquinase synthase	205
<i>aroC</i>	Aromatic	210	Dehydroquinase dehydratase	119, 205
<i>aroD</i>	Aromatic	230	Shikimate dehydrogenase	4, 117, 119
<i>aroE</i>	Aromatic	210	3-Enolpyruvylshikimate-5-phosphate synthase	205, 208
<i>aroF</i>	Aromatic	210	Chorismate synthase	119, 205
<i>aroG</i>	Aromatic	270	Chorismate mutase, isozyme 3	119, 205
<i>aroH</i>	Aromatic	210	Chorismate mutase, isozymes 1 and 2	171, 205
<i>aroI</i>	Aromatic	25	Shikimate kinase	165
<i>aroJ</i>	Aromatic		Tyrosine and phenylalanine; see <i>hisH</i>	207
<i>asaA</i>	Arsenate	230	Arsenate resistance	1,2

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>asaB</i>	Arsenate	57	Arsenate resistance, (derived from <i>B. subtilis</i> W23)	3
<i>aspA</i>	Aspartate		Pyruvate carboxylase, see <i>pycA</i>	33, 117
<i>aspB</i>	Aspartate	200	Aspartate aminotransferase	117
<i>aspH</i>	Aspartate	215	Constitutive aspartase	130, 313
<i>aspT</i>	Aspartate	92	Aspartate transport	312, 313
<i>ath</i>	Adenine-thiamine	55	Adenine-thiamine requirement	328
<i>attSPβ</i>	Attachment	190	Integration site for phage SPβ	330
<i>attSPO2</i>	Attachment	10	Integration site for phage SPO2	133, 273
<i>attφ3T</i>	Attachment		Integration site for phage φ3T, probably maps between <i>kauA</i> and SPβ	318
<i>attφ105</i>	Attachment	245	Integration site for phage φ105	223, 254
<i>azi</i>	Azide	310	Resistance to sodium azide	J. Hoch (unpublished data)
<i>azc</i>	Azetidine carboxylate	25	Resistant to azetidine-2-carboxylic acid; <i>azc</i> 90% cotransformed with <i>arol</i>	82
<i>azlA</i>	Azaleucine	250	4-Azaleucine resistance, derepressed leucine biosynthetic enzymes	310
<i>azlB</i>	Azaleucine	240	4-Azaleucine resistance	310, 311
<i>azpA</i>	Azopyrimidine		Resistance to azopyrimidines; alteration of DNA polymerase III, see <i>polC</i>	49
<i>azpB</i>	Azopyrimidine	330	Resistance to azopyrimidines	39
<i>betR</i>	Betacin	127	Resistant to betacin produced by SPβ lysogens	106
<i>bfmA</i>	Branched fatty acid	126	Lacks branched-chain keto acid dehydrogenase; isolated in <i>aceA</i> strain and not separated genetically from <i>aceA</i>	26, 316
<i>bfmB</i>	Branched fatty acid	216	Requires branched-chain fatty acid, valine, or isoleucine; maps between <i>strC</i> and <i>lys</i>	26, 316
<i>bioA</i>	Biotin	270	7-Oxo-8-aminopelargonate: 7,8-diaminopelargonate aminotransferase	220

(continued)

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>bioB</i>	Biotin	270	Biotin synthetase	220
<i>bio112</i>	Biotin	270	Early defect in biotin synthesis	220
<i>bry</i>	Bryamycin		Bryamycin (thiostrepton) resistance, maps in ribosomal protein	102, 273
<i>bsr</i>	Restriction, modification		Restriction, modification by <i>B. subtilis</i> strain R; see <i>hsrA</i>	294
<i>but</i>		NM	5-Bromouracil-tolerant	22
<i>cafA</i>	Caffeine	46	Caffeine-resistant	40
<i>cafB</i>	Caffeine	359	Caffeine-sensitive; also sensitive to nalidixic acid	40
<i>cal</i>	Chalcomycin	12	Chalcomycin-resistant	264
<i>cam-2</i>	Chloramphenicol	45	Resistant to chloramphenicol	12
<i>carA</i>	Carbamoyl phosphate	100	Subunit of carbamoyl phosphate synthase	202
<i>carB</i>	Carbamoyl phosphate	100	Subunit of carbamoyl phosphate synthase	202
<i>catA</i>	Catabolite resistance	75	Hyperproduction of extracellular proteases, can sporulate in presence of glucose, probably same as <i>scoC</i> and possibly <i>hpr</i>	112, 137, 165, 190
<i>cdd</i>		NM	Deoxycytidine-cytidine deaminase	245
<i>che</i>	Chemotaxis	155	Chemotaxis	215
<i>cheR</i>	Chemotaxis	205	Chemotactic methyltransferase	215
<i>citB</i>	Citric acid cycle	177	Aconitate hydratase	255, 329
<i>citC</i>	Citric acid cycle	260	Isocitrate dehydrogenase	120, 255
<i>citF</i>	Citric acid cycle		Succinate dehydrogenase, now split into three loci; <i>sdhA</i> , <i>sdhB</i> , and <i>sdhC</i>	105, 120, 255
<i>citG</i>	Citric acid cycle	295	Fumarate hydratase	255
<i>citH</i>	Citric acid cycle	260	Malate dehydrogenase	86
<i>citK</i>	Citric acid cycle	185	α -Ketoglutarate dehydrogenase complex, enzyme E1	116, 255
<i>citL</i>	Citric acid cycle	125	Lipoamide dehydrogenase (nicotinamide adenine dehydrogenase, reduced form) E3 component of both pyruvate dehydrogenase and α -ketoglutarate dehydrogenase complexes	116

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>citM</i>	Citric acid cycle	181	Dehydrolipoyltransuccinylase E ₃ component of 2-ketoglutarate dehydrogenase complex; closely linked to <i>citK</i>	69
<i>cml</i>	Chloramphenicol		Chloramphenicol resistance, caused by mutations in one of at least five 50S ribosomal proteins	218
<i>com-9</i>	Competence	280	Poorly competent; defective in DNA binding	66
<i>com-30</i>	Competence	79	Poorly competent; defective in DNA binding	66
<i>com-71</i>	Competence	UC	Poorly competent; defective in DNA binding; may be <i>spo0A</i>	66
<i>com-104</i>	Competence	340	Poorly competent; binds DNA, but little enters the cells	66
<i>cpsX</i> <i>crk</i>		NM	See <i>abrB</i> Cytidine kinase	245
<i>crsA</i>	Catabolite-resistant sporulation	226	Carbon source-resistant sporulation; resistant to novobiocin and acridine orange during sporulation; mutation in <i>rpoD</i> ; has similar phenotype to <i>rut</i> mutations	265, 287
<i>crsB</i>	Catabolite-resistant sporulation	55	Requires high glucose for sporulation	287
<i>crsC</i>	Catabolite-resistant sporulation	217	Carbon source-resistant sporulation; maps close to or in <i>spo0A</i>	287
<i>crsD</i>	Catabolite-resistant sporulation	8	Carbon source-resistant sporulation	287
<i>crsE</i>	Catabolite-resistant sporulation	10	Carbon source-resistant sporulation; maps in <i>rpoBC</i> operon; <i>rjm-11</i> suppresses <i>crsE</i>	287, 288
<i>crsF</i>	Catabolite-resistant sporulation	119	Carbon source-resistant sporulation	287
<i>cys</i>	Cysteine		Cysteine sensitivity; see <i>cysA</i>	145
<i>ctrA</i>	Cytidine requirement	325	Requirement for cytidine in the absence of ammonium ion	165, 329
<i>cyc</i> <i>cym</i>	Cysteine-methionine	95	D-Cycloserine-resistant Requirement for cysteine or methionine; see <i>cysA</i>	81 145, 225

(continued)

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>cysA</i>	Cysteine	11	Cysteine requirement, serine transacetylase; a complex locus consisting of <i>css</i> (cysteine sensitivity), <i>cym</i> (cysteine or methionine), <i>hts</i> (hydrogen sulfide excretion), and <i>cysA</i>	145
<i>cysB</i>	Cysteine	300	Cysteine requirement	60
<i>cysC</i>	Cysteine	140	Cysteine, methionine, sulfite, or sulfide requirement	117, 325
<i>cyt</i>	Cytidine	284	Requires cytidine	126
<i>dal</i>	D-Alanine	40	D-Alanine requirement; alanine racemase	62, 73
<i>dapE</i>	Diaminopimelic acid	124	N-Acetyl-LL-diaminopimelic acid ligase	35
<i>dcd</i>		NM	Deoxycytidine 5'-monophosphate deaminase	198
<i>dck</i>		NM	Deoxycytidine/deoxyadenosine kinase	197, 245
<i>ddd</i>		NM	Deoxycytidine kinase	245
<i>ddl</i>		36	D-Alanyl-D-alanine ligase	35
<i>deoA</i>		NM	Thymidine phosphorylase	180
<i>dfrA</i>	Dihydrofolate	194	Dihydrofolate reductase; coordinately regulated with <i>thyB</i>	204
<i>din</i>	Deoxyribonuclease inhibitor	120	Deoxyribonuclease inhibitor	233
<i>divI</i>	Division	143	Temperature-sensitive cell division, formerly <i>divD</i>	300
<i>divII</i>	Division	320	Temperature-sensitive cell division, formerly <i>divC</i>	300
<i>divIVA</i>	Division	144	Minicell production	243
<i>divIVB</i>	Division	245	Minicell production	243
<i>divIVC</i>	Division	15	Minicell production, formerly <i>divA</i>	300
<i>divV</i>	Division	285	Temperature-sensitive cell division, formerly <i>divB</i>	300
<i>dnaA</i>	DNA	160	DNA synthesis; ribonucleotide reductase	13, 18, 146, 181, 246
<i>dnaB</i>	DNA	255	DNA synthesis, initiation of chromosome replication, probably more than one gene	132, 146, 181, 185
<i>dnaC</i>	DNA	355	DNA synthesis	11, 146, 181
<i>dnaD</i>	DNA	200	DNA synthesis, initiation of chromosome replication	146, 181
<i>dnaE</i>	DNA	230	DNA synthesis	146, 181

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>dnaF</i>	DNA		DNA synthesis, DNA polymerase III; see <i>polC</i>	146, 181
<i>dnaG</i>	DNA	0	DNA synthesis	146, 181
<i>dnaH</i>	DNA	0	DNA synthesis	146, 181, 298
<i>dnaI</i>	DNA	250	DNA synthesis	146, 181
<i>dnaA</i> (Ts)	DNA	350	DNA synthesis	100, 181
<i>dnaB</i> (Ts)	DNA	350	DNA synthesis	100, 181
<i>dna8132</i>	DNA	0	DNA synthesis, initiation of chromosome replication	100, 103, 298
<i>dpa</i>	Dipicolinic acid	148	Requires dipicolinic acid for heat-resistant spores, linked to <i>pyrA</i>	14
<i>dra</i>		339	Deoxyriboaldolase	286
<i>drm</i>		182	Phosphodeoxyribomutase	286
<i>dst</i>	Distamycin	232	Resistant to distamycin and acriflavin	268
<i>D-tyr</i>	D-Tyrosine		Resistance to D-tyrosine; maps within the <i>tyrA</i> locus; see <i>tyrA</i>	43
<i>ebr</i>	Ethidium bromide	325	Ethidium bromide resistance	22
<i>ecp</i>		204	Resistant to 2-amino-5-ethoxycarbonylpyrimidine-4(297H)-one	242
<i>efg</i>	Elongation factor G	10	Elongation factor G	5, 61, 154
<i>ery</i>	Erythromycin		Erythromycin resistance, ribosomal protein L22; see <i>rplV</i>	291, 292
<i>estB</i>	Esterase	294	Esterase B defect	111
<i>fdpA</i>	Fructose diphosphate	344	Fructose-bisphosphatase	76
<i>fibA</i>		310	Macrofiber formation; weak linkage to <i>hisA</i>	260
<i>fibB</i>		295	Macrofiber formation	260
<i>flaA</i>	Flagella	145	Defect in flagellar synthesis, autolysin-deficient	94, 232
<i>flaB</i>	Flagella	149	Defect in flagellar synthesis	94, 232
<i>flaC</i>	Flagella	315	Defect in flagellar synthesis	94, 232
<i>flaD</i>	Flagella	219	Defect in flagellar synthesis, autolysin-deficient	232
<i>fruA</i>	Fructose	120	Fructose transport	87
<i>fruB</i>	Fructose	120	Fructose-1-phosphate kinase	87
<i>fruC</i>	Fructose	51	Fructokinase	87, 89
<i>ftt</i>		208	Fluorotryptophan resistance	20

(continued)

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>fumR</i>	Fumarase	295	Regulation of fumarate hydratase	J. Hoch (unpublished data)
<i>fun</i>		12	Close to, or in, <i>rpsL</i>	108
<i>furA</i>	5-Fluorouracil	135	Resistance to 5-fluorouracil	60
<i>furB</i>	5-Fluorouracil	41	Fluorouracil resistance	328
<i>furC</i>	5-Fluorouracil	325	Resistance to 5-fluorouracil in the presence of uracil	C. Anagnostopoulos (unpublished data)
<i>furE</i>	5-Fluorouracil	325	Resistance to 5-fluorouracil in the presence of uracil	S. Zahler (unpublished data)
<i>furF</i>	5-Fluorouracil	160	Resistance to 5-fluorouracil in the presence of uracil	56
<i>fus</i>	Fusidic acid		Fusidic acid resistance; see <i>efg</i>	154
<i>gca</i>	Glucosamine	NM	L-Glutamine-D-fructose-6-phosphate amino-transferase	74
<i>gdh</i>	Glucose dehydrogenase	32	Structural gene for glucose dehydrogenase, cloned on phage vector	45, 303
<i>gerA</i>	Germination	289	Germination-defective, defective in germination response to alanine and related amino acids, consists of at least three genes	193, 194, 196, 271 195, 257
<i>gerB</i>	Germination	314	Germination-defective, defective in germination response to the combination of glucose, fructose, asparagine, and KCl	194, 195, 271
<i>gerC</i>	Germination	201	Germination-defective, temperature-sensitive germination in alanine; has not been separated from linked mutations in original isolate	194, 195, 271
<i>gerD</i>	Germination	16	Germination-defective, defective germination in range of germinants	194, 195, 229, 271
<i>gerE</i>	Germination	253	Germination-defective, defective germination in range of germinants, may be a spore coat defect	192, 194, 195, 271

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>gerF</i>	Germination	301	Germination-defective, defective germination in a range of germinants	194, 195, 229, 271
<i>gerG</i>	Germination	294	Germination-defective, mutant lacks phosphoglycerate kinase (P _{gk}) activity; germinates poorly in alanine; sporulates poorly	75, 194, 235, 271
<i>gerH</i>	Germination	246	Defective germination in a range of germinants	229
<i>gerI</i>	Germination	296	Defective germination in a range of germinants	229
<i>gerJ</i>	Germination	206	Defective germination in a range of germinants; allele <i>gerJ51</i> (also called <i>tzm</i>) is present in many laboratory strains	309
<i>gerK</i>	Germination	32	Defective germination response to glucose	135
<i>glnA</i>	Glutamine	167	Glutamine synthetase structural gene	25, 56, 70, 83, 244
<i>glpD</i>	Glycerol phosphate	75	Glycerol-3-phosphate dehydrogenase	167
<i>glpK</i>	Glycerol phosphate	75	Glycerol kinase	167
<i>glpP</i>	Glycerol phosphate	75	Pleiotropic glycerol mutant	167
<i>glpT</i>	Glycerol phosphate	15	Fosfomycin-resistant, glycerol phosphate transport defect	152
<i>gltA</i>	Glutamate	180	Glutamate or aspartate requirement, glutamate synthase	56, 117, 199
<i>gltB</i>	Glutamate	180	Glutamate synthase	199
<i>glyA</i>	Glycine	210	Glycine requirement	150–152
<i>glyB</i>	Glycine	75	Glycine requirement	103
<i>glyC</i>	Glycine	320	Glycine requirement	39
<i>gnt</i>	Gluconate	344	Gluconate kinase and permease genes, inability to use gluconate as carbon source	76, 77
<i>groEL</i>		NM	Phage growth	41
<i>gtaA</i>		310	Glucosylation of teichoic acid, lacks uridine-diphosphate-glucose-poly(glycerolphosphate)- α -glucosyltransferase	165, 324
<i>gtaB</i>		310	Glucosylation of teichoic acid	324

(continued)

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>gtaC</i>		75	Glycosylation of teichoic acid	324
<i>gtaC</i>		75	Glucosylation of teichoic acid, lacks phosphoglucomutase	324
<i>guaA</i>	Guanine	0	Inosine monophosphate dehydrogenase	298
<i>guaB</i>	Guanine	50	Guanine requirement	J. Hoch (unpublished data)
<i>guaC</i>	Guanine	NM	Guanosine monophosphate reductase	E. Freese (unpublished data)
<i>guaF</i>	Guanine	NM	Hypoxanthine-guanine phosphoribosyltransferase	64
<i>guaP</i>	Guanine	NM	Inosine-guanosine phosphorylase	64
<i>gutA</i>	Glucitol	50	D-Glucitol permease	42, 89
<i>gutB</i>	Glucitol	50	D-Glucitol dehydrogenase	42, 89
<i>gutR</i>	Glucitol	50	Constitutive synthesis of D-glucitol permease and D-glucitol dehydrogenase, regulatory gene for glucitol catabolism	42, 89
<i>gyrA</i>	Gyrase	357	DNA gyrase (<i>nalA</i>)	39, 88, 160
<i>gyrB</i>	Gyrase	354	DNA gyrase (<i>novA</i>)	39, 160
<i>hag</i>	Flagella	315	Flagellar antigen	94, 176
<i>hds</i>		5	Pleiotropic extragenic suppressors of DNA mutations	269
<i>hemA</i>	Heme biosynthesis	245	δ -Aminolevulinate synthase	153
<i>hemB</i>	Heme biosynthesis	245	Phorphobilinogen synthase	21
<i>hemC</i>	Heme biosynthesis	245	Phorphobilinogen deaminase	21
<i>hemD</i>	Heme biosynthesis	245	Uroporphyrinogen III cosynthase	188
<i>hemE</i>	Heme biosynthesis	90	Uroporphyrinogen decarboxylase	187
<i>hemF</i>	Heme biosynthesis	9	Coproporphyrinogen oxidase	187
<i>hemG</i>	Heme biosynthesis	90	Ferrochelatase	187
<i>hisA</i>	Histidine	299	Histidine requirement, probable location of all histidine enzymes except <i>hisH</i>	24, 65, 308
<i>hisH</i>	Histidine	205	Histidinol-phosphate aminotransferase, tyrosine, and phenylalanine aminotransferase	1, 207

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>hom</i>	Homoserine	290	Threonine and methionine requirement, deletion lacking homoserine dehydrogenase	C. Anagnostopoulous (unpublished data)
<i>hos</i>		12	Suppresses the temperature-sensitive phenotype of elongation factor G mutants	178
<i>hpr</i>	Protease	76	Overproduction of proteases	112
<i>hsrB</i>	Host-specific restriction	345	Host-specific restriction and modification of <i>B. subtilis</i> IAM 1247; endonuclease <i>BsuB</i> , isoschizomer of <i>Pst</i> I	131, 266
<i>hsrC</i>	Host-specific restriction	60	Host-specific restriction and modification of <i>B. subtilis</i> 1247 II; endonuclease <i>BsuC</i>	131
<i>hsrE</i>	Host-specific restriction	337	Host-specific restriction and modification of <i>B. subtilis</i> IMA 1231; endonuclease <i>BsuE</i> ; isoschizomer of <i>FnuDII</i>	131, 143
<i>hsrF</i>	Host-specific restriction	60	Endonuclease <i>BsuF</i> ; isoschizomer of <i>Hpa</i> II	131, 143
<i>hsrM</i>	Host-specific restriction	47	Host-specific restriction and modification of <i>B. subtilis</i> Marburg, probably identical to <i>nonB</i> ; endonuclease <i>BsuM</i> ; isoschizomer of <i>Xho</i> I	131, 143
<i>hsrR</i>	Host-specific restriction	60	Endonuclease <i>BsuR</i> ; isoschizomer of <i>Hae</i> III	31, 143, 294
<i>hts</i>	Hydrogen sulfide		Excretion of hydrogen sulfide; see <i>cysA</i>	145
<i>hut</i>	Histidine utilization	345	Histidine degradation	44, 286
<i>ifm</i>	Flagella	304	Hypermotility, suppresses <i>flaA</i> and <i>flaD</i> mutations	94, 232
<i>igf</i>		343	Deletion covering <i>iol</i> , <i>gnt fdpA</i> , and <i>hsrB</i> (formerly <i>fdpAl</i>)	76
<i>ilvA</i>	Isoleucine-valine	200	Threonine dehydratase	16, 116
<i>ilvB</i>	Isoleucine-valine	250	Condensing enzyme	16, 310
<i>ilvC</i>	Isoleucine-valine	250	α -Hydroxy- β -keto acid reductoisomerase	16, 310
<i>ilvD</i>	Isoleucine-valine	200	Dihydroxyacid dehydratase	91
<i>inh</i>	Inhibition by histidine		Inhibition by histidine; probably within <i>tyrA</i> locus; see <i>tyrA</i>	205, 208

(continued)

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>iol</i>	Inositol	343	Inability to grow on myo-inositol, possibly myo-inositol dehydrogenase gene	76
<i>kan</i>	Kanamycin	10	Kanamycin resistance, maps in the ribosomal protein cluster	92
<i>kauA</i>	Keto acid uptake	185	Branched-chain α -keto transport	91
<i>kir</i>	Kiromycin		Probably mutation in the structural gene for elongation factor Tu, see <i>tuf</i>	274
<i>ksgA</i>	Kasugamycin	4	High-level kasugamycin resistance	38, 293, 298, 319
<i>ksgB</i>	Kasugamycin	280	Low-level kasugamycin resistance	293
<i>ksgC</i>	Kasugamycin	UC	Fumarate hydratase defective, kasugamycin resistance	J. Hoch (unpublished data)
<i>leuA</i>	Leucine	250	α -Isopropylmalate synthase	16, 60, 310, 311
<i>leuB</i>	Leucine	250	Isopropylmalate isomerase	310
<i>leuC</i>	Leucine	250	β -Isopropylmalate dehydrogenase	16, 310, 311
<i>leuD</i>	Leucine	250	Possibly a subunit of isopropylmalate isomerase	174
<i>lin</i>	Lincomycin	25	Lincomycin resistance	
<i>lpm</i>	Lipiamycin		Lipiamycin resistance; RNA ^d polymerase; see <i>rpoC</i>	93, 102, 279
<i>lpmB</i>	Lipiamycin	286	Lipiamycin resistance	277
<i>lys</i>	Lysine	210	Lysine requirement, diamino pimelate decarboxylase	130, 151
<i>lysS</i>	Lysine	5	Lysyl-transfer-RNA synthetase	241
<i>lyt</i>	Lytic		Autolytic enzymes; see <i>flaA</i>	67, 230
<i>mdh</i>			Malate dehydrogenase, see <i>citH</i>	86
<i>menB</i>	Menaquinone	273	Menaquinone-deficient, multiple aminoglycoside-resistant; dihydroxynaphthoate synthase	184, 290
<i>menC,D</i>	Menaquinone	273	Menaquinone-deficient, multiple aminoglycoside-resistant; blocked in formation of o-succinylbenzoic acid from chorismic acid	290

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>menE</i>	Menaquinone	273	Menaquinone-deficient, multiple aminoglycoside-resistant; o-succinylbenzoyl coenzyme A synthetase	184, 290
<i>metA</i>	Methionine	115	Responds to methionine, cystathionine, or homocysteine	282
<i>metB</i>	Methionine	200	Responds to methionine or homocysteine	10
<i>metC</i>	Methionine	115	Responds to methionine	60
<i>metD</i>	Methionine	95	Responds to methionine	324
<i>mic</i>	Micrococcin		Resistance to micrococcin; see <i>rplC</i>	275
<i>mit</i>	Mitomycin	UC	Resistance to mitomycin C; maps near <i>rplV</i>	140
<i>mpo</i>		220	Membrane protein over production, temperature-sensitive sporulation	177
<i>mtlA</i>	Mannitol	34	Lacks mannitol transport; maps near <i>mtlB</i>	P. Gay (unpublished data)
<i>mtlB</i>	Mannitol	35	Mannitol-1-phosphate dehydrogenase	165
<i>mtr</i>	5-Methyltryptophan	210	Resistance to 5-methyltryptophan, derepression of the tryptophan biosynthetic pathway	122, 124
<i>nalA</i>	Nalidixic acid	355	Resistance to nalidixic; see <i>gyrA</i>	103
<i>narA</i>	Nitrate	320	Inability to use nitrate as a nitrogen source	165, 329
<i>narB</i>	Nitrate	30	Inability to use nitrate as a nitrogen source	60, 329
<i>nea</i>	Neamine		Neamine resistance; see ribosomal protein cluster	93
<i>neo</i>	Neomycin		Neomycin resistance; see ribosomal protein cluster	93, 102
<i>nic</i>	Nicotinic acid	245	Nicotinic acid requirement	115, 151
<i>nonA</i>	Nonpermissive	UC	Permissive for bacteriophage SP10 and ϕ NR2, closely linked to <i>rjm</i>	256
<i>nonB</i>	Nonpermissive	45	Permissive for bacteriophage SP10 and ϕ NR2; see <i>hsrM</i>	256
<i>novA</i>	Novobiocin	0	Resistance to novobiocin, <i>gyrB</i>	102, 298

(continued)

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>nprE</i>	Neutral protease	126	Structural gene for neutral (metallo-) protease	299, 304
<i>nprR</i>	Neutral protease	125	Regulatory gene for neutral protease	299
<i>ole</i>	Oleandomycin		Oleandomycin resistance, see ribosomal protein cluster	93, 102
<i>ordA</i>		NM	Ornithine:2 oxoacid aminotransferase	313
<i>outA</i>	Outgrowth	21	Blocked in outgrowth after RNA, protein, and DNA syntheses have started; previous designation <i>gspIV</i>	7, 79, 229
<i>outB</i>	Outgrowth	28	Blocked in outgrowth before most macromolecular syntheses have started; previous designation <i>gsp-81</i>	8, 80, 229
<i>outC</i>	Outgrowth	27	Blocked in outgrowth after RNA and protein syntheses have started, but before DNA synthesis; previous designation <i>gsp-25</i>	7, 229
<i>outD</i>	Outgrowth	122	Blocked in outgrowth; protein and DNA syntheses reduced; previous designation <i>gsp-1</i>	78, 229
<i>outE</i>	Outgrowth	300	Blocked in outgrowth; RNA synthesis normal, protein synthesis reduced; DNA synthesis prevented; previous designation <i>gsp-42</i>	7, 229
<i>outF</i>	Outgrowth	316	Blocked in outgrowth; RNA and protein syntheses reduced; DNA synthesis prevented; previous designation <i>gsp-4</i>	7, 229
<i>oxr</i>		19	Oxolinic acid-resistant	305
<i>pab</i>	<i>p</i> -Aminobenzoic acid	10	<i>p</i> -Aminobenzoic acid requirement, subunit A of <i>p</i> -aminobenzoate synthase	144
<i>pac</i>	Pactamycin	5	Resistance to pactamycin	102, 298
<i>pap</i>			Hyperproduction of proteases and amylase; see <i>sacQ</i>	283, 323

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>pdp</i>		342	Pyrimidine nucleoside phosphorylase	253
<i>pfk</i>		250	Phosphofructokinase	87, 89
<i>pgk</i>		NM	3'-Phosphoglycerokinase	E. Freese (unpublished data)
<i>phal</i>	Phenylalanine	50	Resistance to phage SP01	165
<i>pheA</i>		245	Phenylalanine requirement, prephenate dehydratase	16
<i>phl</i>	Phleomycin	12	Phleomycin resistance; mutator strain linked to <i>rpsL</i>	129
<i>phoA</i>	Phosphatase	NM	Two adjacent structural genes for alkaline phosphatase in <i>B. licheniformis</i>	128
<i>phoP</i>	Phosphatase	260	Regulation of alkaline phosphatase and alkaline phosphodiesterase	162, 322
<i>phoR</i>	Phosphatase	260	Regulation of alkaline phosphatase	161, 162, 189
<i>phoS</i>	Phosphatase	110	Constitutive alkaline phosphatase	228
<i>phoT</i>	Phosphatase	245	Constitutive alkaline phosphatase	P. Piggot (unpublished data)
<i>pig</i>	Pigment	50	Sporulation-associated pigment	249
<i>polA</i>	Polymerase	260	DNA polymerase A	86, 157
<i>polC</i>	Polymerase	145	DNA polymerase III, azopyrimidine resistance	13, 49, 172, 247
<i>pro</i>	Proline	115	Proline requirement	34, 82
<i>ptg</i>	Peptidoglycan	128	Peptidoglycan biosynthesis	35
<i>ptm</i>		105	Pyrimidine resistance	A. Galizzi (unpublished data)
<i>pts</i>		120	Phosphoenolpyruvate phosphotransferase	88, 211
<i>pupA</i>	Purine	NM	Adenosine phosphorylase	99
<i>pupI</i>		NM	Inosine phosphorylase	99
<i>purA</i>		355	Adenine requirement	213
<i>purB</i>		55	Adenine, guanine, or hypoxanthine requirement	171, 214
<i>purC</i>	Purine	55	Adenine or hypoxanthine requirement	171
<i>purD</i>	Purine	55	Adenine or hypoxanthine requirement	171
<i>purE</i>	Purine	55	Adenine requirement	171
<i>pycA</i>	Pyrimidine	127	Pyruvate carboxylase	33, 117
<i>pyrA</i>		135	Carbamyl phosphate synthetase	234

(continued)

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>pyrB</i>	Pyrimidine	135	Aspartate carbamoyl-transferase	234
<i>pyrC</i>	Pyrimidine	135	Dihydroorotase	234
<i>pyrD</i>	Pyrimidine	135	Dihydroorotate dehydrogenase	234
<i>pyrE</i>	Pyrimidine	135	Orotate phosphoribosyltransferase, also called <i>pyrX</i>	234
<i>pyrF</i>	Pyrimidine	135	Orotidine-5'-phosphate decarboxylase	234
<i>pyrG</i>	Pyrimidine	NM	Cytidine-5'-triphosphate synthetase	245
<i>recA</i>	Recombination	145	Genetic recombination and radiation resistance	115, 181
<i>recB</i>	Recombination	245	Genetic recombination and radiation resistance	115, 181
<i>recC</i>	Recombination	?	Genetic recombination; indirect effect of bacteriophage SPO2 lysogeny; see <i>attSPO2</i>	59, 85, 181
<i>recD</i>	Recombination	5	Genetic recombination and radiation resistance	101, 181
<i>recE</i>	Recombination	157	Genetic recombination and radiation resistance, adenosine triphosphate-dependent nuclease; complemented by <i>E. coli recA</i>	57, 59, 181, 182
<i>recF</i>	Recombination	355	Genetic recombination and radiation resistance	101, 181, 298
<i>recG</i>	Recombination	205	Genetic recombination and radiation resistance	182
<i>recH</i>	Recombination	70	Genetic recombination and radiation resistance, adenosine triphosphate-dependent nuclease	181, 206
<i>recI</i>	Recombination	70	Genetic recombination and radiation resistance	81, 206
<i>recL</i>	Recombination	UC	Genetic recombination and radiation resistance, linked to <i>cysA</i>	59, 181, 182
<i>recM</i>	Recombination	5	Genetic recombination and radiation resistance	59, 81, 182
<i>rec149</i>	Recombination	11	Reduced recombination and high sensitivity to mytomycin C, prevents $\phi 105$ restriction in BsuR ⁺ hosts	231
<i>relA</i>	Relaxed	235	ATP:GTP ϵ 3'-phosphotransferase	276, 289

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>relC</i>	Relaxed		See <i>tsp</i> , <i>rplK</i>	276
<i>relG</i>	Relaxed	13	Defect in glucose uptake	236, 237
<i>rev-4</i>	Reversion	324	Suppressor of some pleiotropic effects (but not asporogeny) of <i>spo0</i> mutations; suppresses effect on sporulation of various drug-resistant mutations; may be same as <i>abrA</i>	262, 263
<i>rfm</i>	Rifampin		Rifampin resistance; RNA polymerase; see <i>rpoB</i>	97, 222
<i>rgn</i>		UC	Improved protoplast regeneration, maps near <i>cysA</i>	6
<i>ribA</i>	Riboflavin	209	Riboflavin requirement	150, 239
<i>ribB</i>	Riboflavin	209	Riboflavin requirement	30, 239
<i>ribC</i>	Riboflavin	222	Riboflavin requirement	30
<i>ribD</i>	Riboflavin	209	Riboflavin requirement	30, 239
<i>ribF</i>	Riboflavin	209	Riboflavin requirement	240
<i>ribG</i>	Riboflavin	209	Riboflavin requirement	240
<i>ribH</i>	Riboflavin	209	Riboflavin requirement	240
<i>ribO</i>	Riboflavin	209	Riboflavin requirement	30, 239
<i>ribT</i>	Riboflavin	209	Riboflavin requirement	240
<i>rna53</i>	RNA synthesis	315	Temperature-sensitive RNA synthesis	248
<i>rodB</i>		243	Cell wall defective	147
<i>rodC</i>		320	Cell wall defective	147
<i>rodD</i>		198	Cell wall defective	P. Piggot (unpublished data)
<i>rplA</i>	Ribosomal protein, large	12	Ribosomal protein BL1, chloramphenicol resistance II	54, 217, 218
<i>rplB</i>	Ribosomal protein, large	12	Ribosomal protein L2 (BL2)	272
<i>rplC</i>	Ribosomal protein, large	12	Ribosomal protein L3 (BL3), probably micrococin resistance	51, 217, 275
<i>rplE</i>	Ribosomal protein, large	12	Ribosomal protein L5 (BL6)	51
<i>rplF</i>	Ribosomal protein, large	12	Ribosomal protein L6 (BL8)	51
<i>rplJ</i>	Ribosomal protein, large	12	Ribosomal protein L10 (BL5)	54
<i>rplK</i>	Ribosomal protein, large	12	Ribosomal protein L11 (BL11), thiostrepton resistance, (<i>relC</i>)	54, 217, 222, 315
<i>rplL</i>	Ribosomal protein, large	12	Ribosomal protein L12 (BL9) chloramphenicol resistance VI	54, 217, 218

(continued)

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>rplO</i>	Ribosomal protein, large	10	Ribosomal protein L15, chloramphenicol resistance III	217, 218
<i>rplQ</i>	Ribosomal protein, large	12	Ribosomal protein L17 (BL15)	272
<i>rplU</i>	Ribosomal protein, large	244	Ribosomal protein L21 (BL20)	53
<i>rplV</i>	Ribosomal protein, large	12	Ribosomal protein L22 (BL17), erythromycin resistance	217, 272, 291
<i>rplX</i>	Ribosomal protein, large	12	Ribosomal protein L24 (BL23)	272
<i>rpmA</i>	Ribosomal protein	244	Ribosomal protein L27 (BL24)	53, 68
<i>rpmD</i>	Ribosomal protein	12	Ribosomal protein L30 (BL27)	50
<i>rpoB</i>	RNA polymerase	10	β Subunit of RNA polymerase, rifampin resistance	97, 278
<i>rpoC</i>	RNA polymerase	10	β' subunit of RNA polymerase, streptolydigin resistance	98, 278
<i>rpoD</i>	RNA polymerase	224	RNA polymerase σ^{43} subunit	238
<i>rpsC</i>	Ribosomal protein, small	12	Ribosomal protein S3 (BS3)	272
<i>rpsD</i>	Ribosomal protein, small	12	Ribosomal protein S4 (BS4)	107, 113
<i>rpsE</i>	Ribosomal protein, small	12	Ribosomal protein S5, spectinomycin resistance	50, 102, 164, 216
<i>rpsF</i>	Ribosomal protein, small	4	Ribosomal protein S6 (BS9)	52
<i>rpsG</i>	Ribosomal protein, small	12	Ribosomal protein S7 (BS7)	54
<i>rpsH</i>	Ribosomal protein, small	12	Ribosomal protein S8 (BS8)	219
<i>rpsI</i>	Ribosomal protein, small	12	Ribosomal protein S9 (BS10)	50
<i>rpsJ</i>	Ribosomal protein, small	12	Ribosomal protein S10 (BS13) (<i>tetA</i>)	272
<i>rpsK</i>	Ribosomal protein, small	12	Ribosomal protein S11 (BS11)	50
<i>rpsL</i>	Ribosomal protein, small	12	Ribosomal protein S12 (BS12) (<i>strA</i>), streptomycin resistance	50, 51, 93, 217
<i>rpsP</i>	Ribosomal protein, small	148	Ribosomal protein S16 (BS17)	52
<i>rpsQ</i>	Ribosomal protein, small	12	Ribosomal protein S17 (BS16)	219

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>rpsS</i>	Ribosomal protein, small	12	Ribosomal protein S19 (BS19)	219
<i>rpsT</i>	Ribosomal protein, small	12	Ribosomal protein S20 (BS20)	216, 217, 219
<i>rrnA</i>	Ribosomal RNA	0	Ribosomal RNA operon	214, 319
<i>rrnB</i>	Ribosomal RNA	275	Ribosomal RNA operon	284
<i>rrnD</i>	Ribosomal RNA	70	Ribosomal RNA operon	R. Rudner (unpublished data)
<i>rrnE</i>	Ribosomal RNA	45	Ribosomal RNA operon	R. Rudner (unpublished data)
<i>rrnF</i>	Ribosomal RNA	NM	Ribosomal RNA operon known to exist but not cloned or mapped	K. Bott (unpublished data)
<i>rrnG</i>	Ribosomal RNA	15	Ribosomal RNA operon	K. Bott (unpublished data)
<i>rrnH</i>	Ribosomal RNA	15	Ribosomal RNA operon	K. Bott (unpublished data)
<i>rrnI</i>	Ribosomal RNA	15	Ribosomal RNA operon	K. Bott (unpublished data)
<i>rrnO</i>	Ribosomal RNA	0	Ribosomal RNA operon	214
<i>rrnR</i>	Ribosomal RNA	240	Ribosomal RNA operon located in <i>leu</i> region of the chromosome	R. Rudner (unpublished data)
<i>rvt</i>	Revertant		Mutations causing same phenotype as <i>rvtA</i> mutations but not mapping in the <i>rvtA</i> region	265
<i>rvtA</i>	Revertant	217	Suppressor of sporulation defect in <i>spo0B</i> , <i>spo0E</i> , <i>spo0F</i> , and <i>spoIIA</i> mutants; may be same as <i>sof-I</i> ; intergenic suppression of <i>spo0</i> mutations; probably maps in <i>spo0A</i>	265
<i>sacA</i>	Sucrose	335	β -Fructofuranosidase	163, 164
<i>sacB</i>	Sucrose	305	Levansucrase	163
<i>sacL</i>	Sucrose	240	Levanase	156
<i>sacP</i>	Sucrose	335	Sucrose transport	164
<i>sacQ</i>	Sucrose	285	Hyperproduction of levansucrase and proteases	155, 163
<i>sacR</i>	Sucrose	305	Constitutive α -fructofuranosidase production	163
<i>sacS</i>	Sucrose	335	Constitutive α -fructofuranosidase production	163
<i>sacT</i>	Sucrose	335	Constitutive α -fructofuranosidase production	163
<i>sacU</i>	Sucrose	310	Regulatory gene for levansucrase	155, 163, 166, 283

(continued)

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>sapA</i>	Sporulation-associated phosphatase	114	Mutations overcome sporulation phosphatase-negative phenotype of early blocked <i>spo</i> mutants	226, 228
<i>sapB</i>	Sporulation-associated phosphatase	56	Mutations overcome sporulation phosphatase-negative phenotype of early blocked <i>spo</i> mutations	228
<i>sas</i>		211	Weak intragenic suppressor mutations of <i>spoIIA</i>	326
<i>scoA</i>		109	Sporulation control, protease and phosphatase overproduction; delayed spore formation	190
<i>scoB</i>		129	Sporulation control, protease and phosphatase overproduction; delayed spore formation	58, 190
<i>scoC</i>		75	Sporulation control	190
<i>scoD</i>		130	Sporulation control	190
<i>sdhA</i>	Succinate dehydrogenase	252	Cytochrome b ₅₅₈ subunit of succinate dehydrogenase	105
<i>sdhB</i>	Succinate dehydrogenase	252	Flavoprotein subunit of succinate dehydrogenase	105
<i>sdhC</i>	Succinate dehydrogenase	252	Iron protein subunit of succinate dehydrogenase	105
<i>ser</i>	Serine	210	Requirement for serine	122
<i>serR</i>	Serine	25	Serine resistance	S. Zahler (unpublished data)
<i>smo</i>	Smooth	295	Smooth/rough colony morphology	94, 147
<i>sof-1</i>		217	Suppressor of sporulation defects in <i>spoOB</i> , <i>spoOE</i> , and <i>spoOF</i> mutants; mutation is an alteration in codon 12 of the <i>spoOA</i> gene; probably same as <i>rvtA</i>	121, 148
<i>spcA</i>	Spectinomycin		Spectinomycin resistance: see <i>rpsE</i>	37, 102, 139
<i>spcB</i>	Spectinomycin	140	Spectinomycin resistance	102, 172
<i>spcD</i>	Spectinomycin	3	Spectinomycin dependence, maps between <i>cysA</i> and <i>purA</i>	109

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>spdA</i>		288	Sporulation derepressed; low pyruvate carboxylase activity	63
<i>spe</i>	Spore photoproduct excision	NM	Endonuclease excising spore photoproducts (formerly <i>ssp-1</i>)	203
<i>spg</i>	Sporangiomycin		Sporangiomycin resistance, 50S ribosomal alteration	19
<i>spoCM</i>	Sporulation	355	Stage 0 sporulation, possibly identical to <i>spoJ</i>	29
<i>spoL1</i>	Sporulation	230	"Decadent" sporulation	15
<i>spoOA</i>	Sporulation	215	Sporulation, mutants blocked at stage 0, mutants exhibit wide variety of pleiotropic phenotypes	90, 118, 120, 186, 331
<i>spoOB</i>	Sporulation	245	Sporulation, mutants blocked at stage 0, mutants have most phenotypes of mutants bearing <i>spoOA</i> mutations	27, 68, 118
<i>spoOC</i>	Sporulation	218	Stage 0 sporulation, mutations with less pleiotropic phenotypes known to be missense alterations in the <i>spoOA</i> gene product	121
<i>spoOD</i>	Sporulation	234	Stage 0 sporulation, single allele resulting in stage 0 block of sporulation, mapped but not further characterized	134
<i>spoOE</i>	Sporulation	120	Stage 0 sporulation, oligosporogenous mutations giving stage 0 block, possibly more than one gene, phenotypes less than <i>spoOA</i> , <i>spoOB</i> , or <i>spoOF</i> mutations	47, 227
<i>spoOF</i>	Sporulation	323	Stage 0 sporulation, DNA sequence contains single open reading frame for protein of 19,055 Da, inhibits sporulation when present in multiple copies	118, 149, 267
<i>spoOG</i>	Sporulation	223	Stage 0 sporulation, single allele resulting in stage	134

(continued)

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>spo0H</i>	Sporulation	11	0 block of sporulation, maps in region of <i>spo0A</i> locus but genetically distinct from <i>spo0A</i> Stage 0 sporulation, locus codes for protein of 22,000 Da determined from sequence of <i>B. licheniformis</i> cloned gene complementing <i>spo0H</i> mutations of <i>B. subtilis</i> , least pleiotropic <i>spo0</i> mutation	118, 224, 227
<i>spo0J</i>	Sporulation	352	Stage 0 sporulation, locus consisting of two alleles yielding phenotype similar to <i>spo0H</i> mutations	127, 298
<i>spo0K</i>	Sporulation	101	Stage 0 sporulation, maps close to tryptophanyl transfer RNA synthetase gene	47, 227
<i>spo0L</i>	Sporulation	106	Stage 0 sporulation, uncharacterized allele giving <i>spo0</i> phenotype, maps near <i>spo0K</i> but genetically distinct	J. Hoch (unpublished data)
<i>spoIIA</i>	Sporulation	211	Stage II sporulation, blocked at stage II of sporulation, DNA sequence has three adjacent open reading frames coding for proteins of 13, 16, and 22-kDa; 22-kDa one has homology to <i>rpoD</i>	47, 72, 134, 170
<i>spoIIB</i>	Sporulation	244	Stage II sporulation	47, 227
<i>spoIIC</i>	Sporulation	296	Stage II sporulation	227
<i>spoIID</i>	Sporulation	316	Stage II sporulation	227
<i>spoIIE</i>	Sporulation	10	Stage II sporulation	227
<i>spoIIF</i>	Sporulation	120	Stage II sporulation	127, 227
<i>spoIIG</i>	Sporulation	135	Stage II sporulation, DNA sequence as homology with <i>rpoD</i> gene	227, 285
<i>spoIIIA</i>	Sporulation	220	Stage III sporulation, blocked at stage III of sporulation	224, 227
<i>spoIIIB</i>	Sporulation	221	Stage III sporulation, blocked at stage III of sporulation	224, 227

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>spoIIIC</i>	Sporulation	227	Stage III sporulation, blocked at stage III of sporulation; possibly the same as <i>spoIVC</i>	134, 227
<i>spoIIID</i>	Sporulation	302	Stage III sporulation, blocked at stage III of sporulation	134, 227
<i>spoIIIE</i>	Sporulation	142	Stage III sporulation, blocked at stage III of sporulation	134, 227
<i>spoIIIF</i>	Sporulation	239	Stage III sporulation, blocked at stage III of sporulation; map order <i>nic-recB-spoIIIF-spoVB</i>	159
<i>spoIVA</i>	Sporulation	204	Stage IV sporulation, linked to <i>trpC</i> by transformation	227
<i>spoIVB</i>	Sporulation	213	Stage IV sporulation, may be allele of <i>spo0A</i>	47, 227
<i>spoIVC</i>	Sporulation	227	Stage IV sporulation, contains at least two cistrons, linked to <i>aroD</i> by transformation	55, 227, 229
<i>spoIVD</i>	Sporulation	233	Stage IV sporulation	127, 227
<i>spoIVE</i>	Sporulation	234	Stage IV sporulation	134, 227
<i>spoIVF</i>	Sporulation	242	Stage IV sporulation, linked to <i>spo0B</i> by transformation	47, 159, 227
<i>spoIVG</i>	Sporulation	97	Stage IV sporulation	224, 227
<i>spoVA</i>	Sporulation	211	Stage V sporulation, transcribed as polycistronic unit with open reading frames for proteins of 23, 15, 16, 36, and 34 kDa	71, 227
<i>spoVB</i>	Sporulation	239	Stage V sporulation	127, 159
<i>spoVC</i>	Sporulation	7	Stage V sporulation, cloned on plasmid vectors	200
<i>spoVD</i>	Sporulation	133	Stage V sporulation, linked to <i>spoVE</i> by transformation	47, 127
<i>spoVE</i>	Sporulation	133	Stage V sporulation	47, 127, 227
<i>spoVF</i>	Sporulation	148	Stage V sporulation, probably the same as <i>dpa</i> ; mutants form octanol- and chloroform-resistant, heat-sensitive spores; form heat-resistant spores in presence of dipicolinic acid	14, 227, 229

(continued)

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>spoVG</i>	Sporulation	6	Stage V sporulation, previously called 0.4-kilobase gene, transcription turned on within 30 min of start of sporulation	96, 252
<i>spoVH</i>	Sporulation	251	Stage V sporulation	114
<i>spoVJ</i>	Sporulation	250	Stage V sporulation	114
<i>spoVIA</i>	Sporulation	255	Blocked at stage VI of sporulation; map order <i>argA-spoVIA-gerE-leuA</i>	141
<i>spoVIB</i>	Sporulation	247		142
<i>spoL</i>		227	"Decadent" sporulation	15
<i>sprA</i>		NM	Derepression of homoserine kinase, homoserine dehydrogenase, and the minor threonine dehydratase (<i>tdm</i>)	301
<i>sprB</i>		290	Partial suppression of isoleucine requirement allows threonine dehydratase <i>sprA</i> mutants to grow in minimal medium, maps near <i>tdm</i> locus; see <i>tdm</i>	302
<i>sprE</i>			See <i>aprE</i>	
<i>srm</i>		12	Modifies resistance of <i>spcA</i> strains	38
<i>ssa</i>		217	Alcohol-resistant sporulation, maps close to <i>spoOA</i> , <i>rvt</i> mutations have same phenotype	23, 265
<i>ssp-1</i>	See <i>spe</i>			
<i>sspA</i>	Spore-specific protein	265	One member of multigene family coding for small acid-soluble spore protein	P. Setlow (unpublished data)
<i>sspB</i>	Spore-specific protein	65	Same as <i>sspA</i>	P. Setlow (unpublished data)
<i>sspC</i>	Spore-specific protein	180	Same as <i>sspA</i>	P. Setlow (unpublished data)
<i>sspD</i>	Spore-specific protein	120	Same as <i>sspA</i>	P. Setlow (unpublished data)
<i>std</i>	Streptolydigin		Streptolydigin resistance, RNA polymerase; see <i>rpoC</i>	98, 278
<i>strA</i>	Streptomycin		Streptomycin resistance; see <i>rpsL</i>	93
<i>strB</i>	Streptomycin	130	Streptomycin resistance	280

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>strC</i>	Streptomycin	220	Streptomycin uptake deficient, possible cytochrome oxidase regulator	183, 280
<i>sul</i>	Sulfonilamide	10	Sulfonilamide resistance	133, 144
<i>suA20</i>	Suppressor	145	Suppressor of <i>recH</i> mutations with increased ATP-dependent deoxyribonuclease	158
<i>sup3</i>	Suppressor	30	Suppressor transfer RNA	110
<i>sup44</i>	Suppressor	29	Suppressor transfer RNA	169
<i>tag</i>		309	Cell wall synthesis	28, 147
<i>tal</i>	β -Thienylalanine	239	Resistant to β -thienylalanine	46
<i>tdm</i>		290	Minor threonine dehydratase	302
<i>ten</i>	Transfection enhancement	245	Constitutive transfection enhancement of SP82 DNA, transformation-defective	D. Green (unpublished data)
<i>tetB</i>	Tetracycline	3	Resistant to tetracycline	317
<i>thiA</i>	Thiamine	70	Thiamine requirement	151
<i>thiB</i>	Thiamine	100	Thiamine requirement	117
<i>thiC</i>	Thiamine	331	Thiamine requirement	S. Zahler (unpublished data)
<i>thrA</i>	Threonine	290	Threonine requirement, homoserine kinase	60, 301
<i>thyA</i>	Thymidine	160	Thymidylate synthetase A	10, 210, 320
<i>thyB</i>	Thymidine	200	Thymidylate synthetase B	10, 210, 320
<i>til</i>	Tilerone	230	Tilerone resistance	J. Hoch (unpublished data)
<i>tkt</i>		NM	Transketolase	258, 259
<i>tmp</i>	Trimethoprim	200	Trimethoprim resistance	307
<i>tmrA</i>	Tunicamycin	25	Tunicamycin resistance, hyperproductivity of extracellular α -amylase	212
<i>tmrB</i>	Tunicamycin	25	Tunicamycin resistance	212
<i>tms12</i>		130	Temperature-sensitive cell division	48
<i>tms26</i>		5	Temperature-sensitive cell division	48, 294
<i>tolA</i>	Tolerance		Tolerance to bacteriophage; see <i>abrB</i>	136, 138
<i>tolB</i>	Tolerance	NM	Tolerance to bacteriophage	136, 138
<i>tre</i>	Trehalose	60	Trehalose	306
<i>trnA</i>	Transfer RNA	0	Genes for Ile and Ala transfer RNAs located between 16S and 23S RNAs in the <i>rrnA</i> operon	

(continued)

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>trnB</i>	Transfer RNA	275	Linked set of transfer RNA genes distal to <i>trnB</i> that contains transfer RNAs for Val, Thr, Lys, Leu, Gly, Leu, Arg, Pro, Ala, Met, Ile, Ser, fMet, Asp, Phe, His, Gly, Ile/Met, Asn, Ser, and Glu	306
<i>trnI</i>	Transfer RNA	15	Linked set of transfer RNA genes including transfer RNAs for Ala, Pro, Arg, Gly, Thr, and Asn located between <i>trnH</i> and <i>trnI</i> , formerly <i>trnH</i>	306
<i>trnO</i>		0	Same transfer RNA genes as <i>trnA</i> but located in <i>trnO</i>	306
<i>trnR</i>	Transfer RNA	240	Linked set of transfer RNA genes thought to be distal to <i>trnR</i> that contains transfer RNAs for Asn, Ser, Glu, Val, Met, Asp, Phe, Thr, Tyr, Trp, His, Gln, Gly, Cys, Leu, and Leu	306
<i>trnY</i>	Transfer RNA	NM	Linked set of transfer RNA genes for Lys, Glu, Asp, and Phe	306
<i>trpA</i>	Tryptophan	205	Tryptophan synthase α	9, 123, 314
<i>trpB</i>	Tryptophan	205	Tryptophan synthase β	9, 123, 314
<i>trpC</i>	Tryptophan	205	Indol-3-glycerol-phosphate synthase	9, 123, 314
<i>trpD</i>	Tryptophan	205	Anthranilate phosphoribosyltransferase	9, 123, 314
<i>trpE</i>	Tryptophan	205	Anthranilate synthase	9, 123, 314
<i>trpF</i>	Tryptophan	205	N-(137'-Phosphoribosyl) anthranilate isomerase	123, 314
<i>trpS</i>	Tryptophan	100	Tryptophanyl-transfer RNA synthase	282
<i>trpX</i>	Tryptophan	10	Glutamine-binding protein common to anthranilate synthase and <i>p</i> -aminobenzoate synthase	144
<i>tsi</i>		50	Temperature-sensitive induction of all known SOS functions	173, 270
<i>tsp</i>	Thiostrepton		Thiostrepton resistance, 50S subunit, maps in ribosomal protein cluster	222

Table I. (Continued)

Gene symbol	Mnemonic	Map position ^a	Phenotype, enzyme deficiency, or other characteristic	References ^b
<i>tsl</i>		145	Temperature-sensitive division	36
<i>ts31</i>		131	Temperature-sensitive division	191
<i>ts39</i>		230	Temperature-sensitive synthesis of phosphatidylethanolamine	168
<i>ts341</i>		312	Temperature-sensitive division	191
<i>ts526</i>		3	Temperature-sensitive division	191
<i>tuf</i>	Tu factor	10	Elongation factor Tu	61
<i>tyrA</i>	Tyrosine	205	Tyrosine requirement, prephenate dehydrogenase	205, 208
<i>tzm</i>	Tetrazolium	205	Tetrazolium reaction; probably an allele of <i>gerJ</i>	298, 309
<i>udk</i>	Uridine kinase	NM	Uridine kinase, also lacks cytidine kinase, mutant resistant to fluorouridine	209
<i>upp</i>		NM	Uracil phosphoribosyltransferase	209
<i>urg</i>		NM	<i>N</i> -Glycosidase	175
<i>urs</i>		100	Uracil sensitivity, arginine-specific carbamoyl-phosphate synthase; same as either <i>carA</i> or <i>carB</i>	R. Switzer (unpublished data)
<i>uvrA</i>	Ultraviolet repair	310	Excision of ultraviolet light-induced pyrimidine dimers in DNA	115, 165, 203
<i>uvrB</i>	Ultraviolet repair	250	Excision of ultraviolet light-induced pyrimidine dimers in DNA	203
<i>vas</i>			Valine sensitivity; maps within threonine dehydratase locus; see <i>ilvA</i>	203
VS		12	Virginiamycin (VS component) resistance	250
<i>xhi</i>		112	Heat-inducible PBS-X	32, 34
<i>xlt</i>		115	Induced PBS-X bacteriophage lack tails	84
<i>xynA</i>		48	Extracellular β -xylanase	251
<i>xynB</i>		48	Cell-associated β -xylooxidase	251

^aUC, map position not fully defined; NM, not mapped.

^bSee reference list (pp. 394–406).

^cATP, adenosine triphosphate.

^dRNA, ribonucleic acid.

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